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REPORT #2

"Host Defense Against Opportunist Microorganisms Following Trauma"

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D.
William A. Altemeier, M.D.
H. Stephen Bjornson, M.D., Ph.D.

JUNE, 1977

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Washington, D.C. 20314

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University of Cincinnati

Cincinnati, Ohio 45221

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Studies were performed to determine the effects of septicemia on humoral components of host defense in burned patients. Conversion of C3 by inulin, total hemolytic complement (CH50), and immunochemical levels of C1, C4, C2, C3, C5, factor B, C3b inactivator (KAF), and properdin were measured in fifteen patients with severe thermal injury during nine weeks postburn. Five of the patients had one or more septic episodes as documented by positive blood cultures and clinical findings; another patient had one positive blood culture which was (continued)

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unassociated with fever or any other clinical findings associated with septicemia. A change in the complement profile was not observed in this patient during the transient bacteremia, however consumption of components of the classical complement pathway occurred prior to and during the septic episodes in the other infected patients. Alternative pathway activity was also decreased in the burned patients during septic episodes, however the data indicated that this pathway was inhibited rather than activated. In one patient, consumption of components of the classical complement pathway occurring during septicemia decreased the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of E. coli. In the other patients, consumption of classical pathway components did not reduce the opsonic capacity of the patients' sera for their infecting microorganisms. All of the microorganisms isolated from the burned patients were not susceptible to complement mediated lysis and were phagocytosed and killed intracellularly by human leukocytes only in the presence of human serum. The only exceptional microorganisms were the C. albicans strains which were not phagocytosed and killed intracellularly by leukocytes even in the presence of high concentrations of normal human serum.

Data from the nine burned patients who did not develop septicemia was similar to the data obtained on our previously studied groups of non-septic patients with similar burn sizes. Preliminary evidence was provided to suggest that reduction in C3 conversion via the alternative pathway occurring in burned patients during the tenth to 60th postburn days was caused by an elevation of a normal regulatory protein of the complement system. Inhibition of the alternative pathway in the burned patients caused preferential utilization of the classical pathway, a new finding of considerable biological importance.

To determine if the changes in complement components and immunoglobulins which were observed in the septic and non-septic burned patients were unique to patients with burn injury, levels of components of the alternative and classical complement pathways and immunoglobulins were measured in the sera of ten patients with blunt or penetrating abdominal trauma and in ten septic patients without trauma. Abnormalities of both the alternative and classical complement pathways and of immunoglobulin M were demonstrated in the trauma patients. No reduction in classical or alternative pathway activity or of immunoglobulin levels was demonstrated in the septic medical patients, suggesting that complement consumption in the septic burned patients resulted from synergism between the infection and the trauma.

Experiments were also performed to investigate the humoral mechanisms which are operative against gram-negative aerobic and anaerobic opportunist microorganisms which cause serious infection in trauma patients. Intact cells of E. coli 075, wild-type S. minnesota, and clinical isolates of P. aeruginosa and P. mirabilis were found to be capable of efficiently activating the alternative pathway. Utilizing washed cells of cell wall mutants of S. minnesota, the polysaccharide portion of the lipopolysaccharide was shown to be responsible for alternative pathway activation, whereas the lipid A moiety was responsible for activation of the classical pathway. In addition, a requirement for immunoglobulin and components of the alternative complement pathway was demonstrated for opsonization of Bacteroides fragilis subspecies fragilis and thetaiotaomicron.

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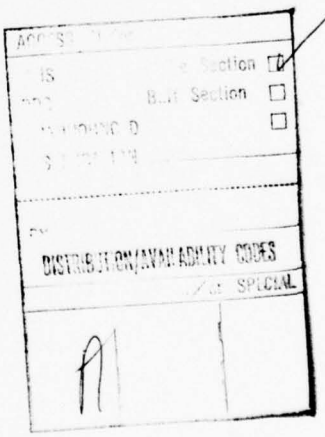
FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.



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Abstract

Studies were performed to determine the effects of septicemia on humoral components of host defense in burned patients. Conversion of C3 by inulin, total hemolytic complement (CH_{50}), and immunochemical levels of C1, C4, C2, C3, C5, factor B, C3b inactivator (KAF), and properdin were measured in fifteen patients with severe thermal injury during nine weeks postburn. Five of the patients had one or more septic episodes as documented by positive blood cultures and clinical findings; another patient had one positive blood culture which was unassociated with fever or any other clinical findings associated with septicemia. A change in the complement profile was not observed in this patient during the transient bacteremia, however consumption of components of the classical complement pathway occurred prior to and during the septic episodes in the other infected patients. Alternative pathway activity was also decreased in the burned patients during septic episodes, however the data indicated that this pathway was inhibited rather than activated. In one patient, consumption of components of the classical complement pathway occurring during septicemia decreased the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of E. coli. In the other patients, consumption of classical pathway components did not reduce the opsonic capacity of the patients' sera for their infecting microorganisms. All of the microorganisms isolated from the burned patients were not susceptible to complement mediated lysis and were phagocytosed and killed intracellularly by human leukocytes only in the presence of human serum. The only exceptional microorganisms were the C. albicans strains which were not phagocytosed and killed intracellularly by leukocytes even in the presence of high concentrations of normal human serum.

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To determine if the changes in complement components and immunoglobulins which were observed in the septic and non-septic burned patients were unique to patients with burn injury, levels of components of the alternative and classical complement pathways and of immunoglobulins were measured in the sera of ten patients with blunt or penetrating abdominal trauma and in ten septic patients without trauma. Abnormalities of both the alternative and classical complement pathways and of immunoglobulin M were demonstrated in the trauma patients. No reduction in classical or alternative pathway activity or of immunoglobulin levels was demonstrated in the septic medical patients, suggesting that complement consumption in the septic burned patients resulted from synergism between the infection and the trauma.

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requirement for immunoglobulin and components of the alternative complement pathway was demonstrated for opsonization of Bacteroides fragilis subspecies fragilis and thetaiotaomicron.

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I. INTRODUCTION

A primary cause of morbidity and mortality in military personnel who have sustained burns, gunshot and high explosive wounds, or crush injuries is microbial infection (1, 2). The widespread prophylactic use of antibiotics has not only failed to decrease the incidence of infection, particularly in the burned patient, but has also contributed to the complexity of the problem through the development of infections caused by antibiotic resistant microorganisms. Pseudomonas aeruginosa, Proteus, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, and Candida albicans are the microorganisms which are most frequently associated with septic complications in the thermally injured patient, and Bacteroides fragilis has assumed a major role in the etiology of peritonitis caused by penetrating abdominal injury. The increased incidence of recovery of penicillin resistant and more recently, clindamycin resistant strains of B. fragilis (3) suggests that these microorganisms may become one of the major infectious problems of the future.

Management of surgical infections has classically involved antibiotic therapy and the meticulous care of the surgical wound. However, in addition to these therapeutic modalities, attention has recently been focused upon defining immunological abnormalities of the host which may predispose him to microbial infection. Studies of alterations of host defense mechanisms in surgical patients have predominantly been carried out in patients with severe thermal injury. The data obtained from these investigations have provided evidence to suggest that neutrophil anti-staphylococcal activity (4), phagocytic function of the reticuloendothelial system (5), cell-mediated immunity (6), serum opsonins (7-9), levels of immunoglobulins (10-14), and classical (9, 15) and alternative (8, 9) complement components are reduced following burn trauma.

The investigation described in this report was undertaken to determine the

cause and significance of alterations of complement and immunoglobulins in burned patients. Studies were initiated to determine if the humoral abnormalities observed in the burned patients were unique to patients with burn trauma or were also observed in patients with other types of trauma or in septic patients without trauma. In addition, experiments were conducted to define the role of antibodies and complement in host defense against P. aeruginosa, E. coli, and B. fragilis.

II. BACKGROUND

A. Changes in Humoral Components of Host Defense Following Burn Injury

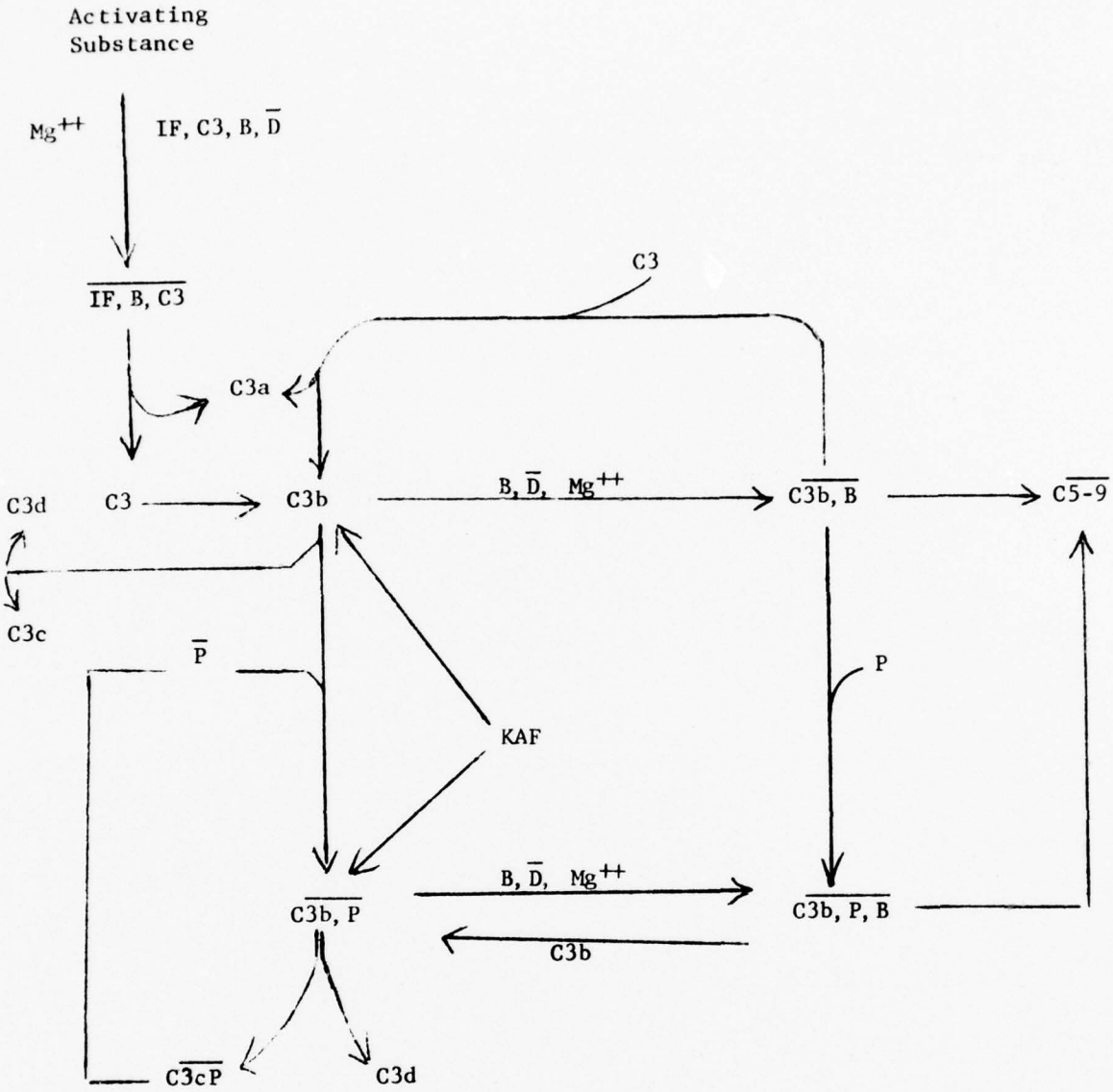
In our previous studies, the competence of the alternative and classical pathways of complement activation was studied in patients following burn injury, and abnormalities of both pathways were demonstrated. The protein interactions involved in classical complement pathway activation have been defined and are widely accepted (16). However, the proteins required for alternative pathway activation are poorly understood and continue to be actively investigated. The most recent evidence suggests that the initial stage in the conversion of C3 by zymosan or inulin requires native C3, factor B, factor $\overline{D}$, and initiating factor, a newly discovered β globulin which reacts immunochemically with C3 nephritic factor (17, 18). C3b, which is generated during C3 conversion, initiates a positive feedback mechanism by modulating the conformation of factor B so that this molecule can be activated by factor $\overline{D}$ (19-21). Properdin, which was originally postulated to be required for formation of the initial C3 convertase (19, 22, 23), has recently been shown to stabilize the enzyme $\overline{C3b, B}$ which is formed by the feedback mechanism (18, 24). Both $\overline{C3b, B}$ and stabilized $\overline{C3b, P, B}$ serve as C3 and C5 convertases, suggesting that properdin functions as a modulator of an already assembled enzyme. The C3 and C5 convertase functions of $\overline{C3b, P, B}$ are inhibited by fluid phase C3b which causes dissociation of factor B. The resultant $\overline{C3b, P}$ enzyme can then be dissociated by KAF which cleaves $\overline{C3b, P}$ to $\overline{C3cP}$ and C3d.

The question of the role of immunoglobulins in activation of the alternative pathway is unanswered. Available evidence indicates that activation by inulin or zymosan may occur in the absence of immunoglobulins (18). On the other hand, antibody of the IgG class has been shown to participate in lysis of measles virus-infected cells (25) and in opsonization of *P. aeruginosa* (26, 27)

and Streptococcus pneumoniae (28) via the alternative pathway. The tentative reaction sequence of the alternative pathway utilizing zymosan or inulin as the activating substance is represented in Figure 1.

The changes in the alternative pathway of complement activation which were shown to occur following burn injury are as follows: a) properdin was reduced in the burn sera during the first 30 days postburn; b) the C3 converting activity of the sera by inulin was reduced after the first 10 days postburn and was normalized by the seventh week; and c) levels of native C3, KAF, and factor B were frequently increased during 6 to 8 weeks postburn. In several of our patients, C3 conversion was also reduced during the initial postburn period. Reduction in the C3 converting activity and properdin concentration was not found to be related to age and correlated with increasing burn size; however, no correlation between the two abnormalities was demonstrated. The finding that C3, KAF, and factor B were elevated in the burn sera suggests that these components are probably acute phase reactants in the thermally injured patient. Patients with the smallest burn sizes had the most striking increase in the levels of C3, KAF, and factor B, probably because they were not losing as much protein through their burn wounds as the patients with the larger burns.

Reduction in the C3 converting activity of the burn sera appeared to be related to the presence of an inhibitor, since it could not be fully corrected by addition of normal human serum. However, it is necessary to demonstrate that addition of a purified factor from burn serum reduces C3 conversion in normal serum before any conclusions regarding the nature of the reduction in C3 conversion can be drawn. The significance of the reduction in properdin following burn injury is difficult to evaluate since properdin has recently been shown to play an ancillary role in the formation of C3 and C5 alternative pathway convertases (18, 24).



IF = initiating factor
B = factor B
D = factor D
KAF = C3b inactivator
P = precursor properdin
P̄ = activated properdin

Fig. 1. Tentative reaction sequence of the alternative complement pathway.

A decrease in serum IgG was also observed in the burn patients during the early postburn period with children under the age of 4 showing the most severe and prolonged deficiency, lasting up to 30 days postburn. In both young and older burn patients, levels of IgA were only reduced during the first 5 days postburn. In all patients, levels of IgM were reduced during the entire 40-day postburn period. Our results confirm those of other investigators who have determined immunoglobulin patterns following burn trauma (10-14), although a more prolonged decrease in IgM level was demonstrated in our study. The absence of a significant difference in immunoglobulin levels in patients with large and small burns and the marked depression in IgM level indicate that exudative loss of protein from the burn surface is probably only partially responsible for the observed abnormalities.

No differences in the occurrence of abnormalities of immunoglobulins or complement were noted in patients with burns caused by flame, immersion scald, or contact with sulfuric acid. However, in the patient with acid burns, the onset of reduction in C3 conversion occurred during the third to fourth postburn week rather than during the second week. In addition, the concentration of properdin in this patient's serum was not restored to normal during the entire study period of 60 days postburn. The difference in the temporal sequence of these abnormalities in relation to burns caused by flame or immersion scald may be related to the type of burn injury. Sulfuric acid does not burn tissue by hyperthermic activity; rather, it coagulates protein by desiccation (29).

A cause and effect relationship could not be established between any of the observed abnormalities of immunoglobulins or complement and the development of septicemia, since only two of the study patients became septic. However, preliminary evidence indicated that consumption of components of the classical and alternative complement pathways was associated with and was possibly caused

by infection. Reduction in the classical complement pathway occurred during the first 10 days postburn in an infant who developed septicemia with S. aureus on the third postburn day, which persisted through the sixth day. Reduction in the classical and alternative pathways of complement activation was also observed in a 39-year-old female who had multiple episodes of Pseudomonas septicemia and candidemia during her clinical course and who died of septic shock. Fearon et al. have demonstrated reduction in components of the classical and alternative complement pathways in a series of medical patients with gram-negative bacteremia associated with shock, and have suggested that biologically active products released during activation of C3 through C9 by the bacteria contributed to development of shock (30).

Abnormalities of immunoglobulins and of the alternative complement pathway were observed in burned patients with and without septicemia. However, reduction in the classical pathway of complement activation was only observed in the burned patients during septicemia. Fjellstrom and Arturson previously demonstrated reduction in C1, C4, C2, and C3 in severely burned patients and suggested that infection might be a possible cause for this complement deficiency (15). A study of the specific components of the classical pathway which are activated in burned patients during septicemia will be a subject of this investigation.

The classical complement pathway was shown to be utilized preferentially during opsonization of E. coli 075. In the presence of an intact alternative pathway C3 convertase, reduction in the opsonic activity of the burn sera for E. coli 075 was only observed when total hemolytic complement was also reduced. However, Jasin previously demonstrated that in agammaglobulinemia serum depleted of C1q, factor B of the alternative complement pathway was required for opsonization of this microorganism (31). Several explanations for these contradictory observations are as follows: a) If properdin were required for opsonization of

E. coli 075, then reduction in the properdin level of the burn sera might divert activation from the alternative to the classical complement pathway. In this regard, we have shown that normal human serum depleted of properdin supports normal opsonization of E. coli 075, suggesting either that properdin is not required for opsonization or that, in the absence of properdin, the classical complement pathway is utilized. b) The classical complement pathway may be activated in the burned patients during opsonization of E. coli 075 because sufficient antibodies are present in the sera which react with the microorganism and activate C1. These antibodies may exist naturally in human sera or may be produced in the burned patients nonspecifically as a result of the administration of P. aeruginosa vaccine. All of the study patients received 25 μg/kg of the vaccine upon admission, 4 and 8 days thereafter, and then at weekly intervals. c) Only in the complete absence of immunoglobulin is the alternative pathway activated by E. coli 075. Studies in this investigation will be undertaken to determine if E. coli 075 can activate the alternative pathway in normal human serum and to compare heat-stabile opsonizing antibodies in normal individuals and in vaccinated and nonvaccinated burned patients.

One of our most important findings was that multiple abnormalities of humoral components of host defense were demonstrated in patients who did not develop systemic infection. This finding emphasizes the importance of the clinical management of the patient in preventing the development of septic complications. If the patient does become septic, consumption of components of the classical and/or alternative pathways of complement activation may be an important mechanism by which infection is perpetuated. Of considerable importance in this investigation will be to address the question of whether reduction in C3 conversion via the alternative complement pathway may also compromise host defense by reducing the opsonic capacity of the serum for a potential infecting

organism which normally utilizes components of this pathway during opsonization. This abnormality occurs during the time when the burned patient is the most susceptible to infection, suggesting that it may be potentially important in predisposition to infection. In addition, it will be important to know if changes in the alternative and classical complement pathways are unique to patients with burn injury or are also observed in patients with other types of trauma and in patients with septicemia without trauma. Of future interest will also be studies concerning the mechanisms by which abnormalities of the humoral factors occur and their relationship to abnormalities of cellular components of host defense.

B. Normal Human Serum Opsonins for P. aeruginosa and E. coli

In the studies which have been reported in the literature regarding participation of the alternative complement pathway in opsonic functions required for in vitro phagocytosis and intracellular killing of bacteria, a requirement for a specific serum protein in opsonization was demonstrated by its ability to restore opsonic activity to serum depleted of that respective protein. This approach has the capability of determining whether or not a serum protein is required for phagocytosis and intracellular killing of a bacterial strain, but is limited by its inability to determine all of the proteins required for these events. This determination can only be made by adding combinations of purified proteins to bacteria and determining whether or not phagocytosis and intracellular killing of the bacteria by leukocytes will occur; this is the experimental approach which has been utilized in our studies.

Properdin and factor B have been obtained from normal human serum in partially purified form. By alkaline polyacrylamide disc gel electrophoresis, both protein preparations were found to be only contaminated with gamma globulin. We have also had encouraging preliminary results in preparing factor $\bar{D}$ depleted

human serum for the functional testing of fractions potentially containing factor $\overline{D}$. A subject of this investigation will be the purification of human factor $\overline{D}$.

Normal IgG has been successfully purified by ammonium sulfate fractionation and chromatography on DEAE cellulose. After a second fractionation on TEAE cellulose (Cellex T), further purification was obtained. By immunoelectrophoretic analysis using antiserum to whole human serum, normal IgG preparations contained a single line migrating in the gamma region. By radial immunodiffusion, the preparations contained no IgM or properdin, and only trace amounts of IgA.

The opsonic activities for E. coli 075 and P. aeruginosa of the partially purified preparations of C3, factor B, properdin, and normal IgG have been tested. Thus far, the results have demonstrated no reduction in bacterial counts by normal human leukocytes in the presence of physiological concentrations of the proteins when tested either individually or in combination. These findings are preliminary and only begin to approach the complex problem of determining which components of the alternative pathway participate in opsonization of the microorganisms and the requirement for normal IgG.

C. In Vitro Interaction of Bacteroides fragilis and Fusobacterium Mortiferum with Human Serum and Leukocytes

Prior to our study, no information was available regarding specific humoral and cellular host defense mechanisms against gram-negative anaerobic microorganisms. Phagocytosis and killing by polymorphonuclear leukocytes (PMNS) has been shown to be of primary importance in host defense against extracellular and facultative aerobic microorganisms. Serum bactericidal activity against gram-negative aerobic enteric bacilli is also well recognized. The purpose of our study was to determine if these bactericidal mechanisms were operative against strains of B. fragilis and F. mortiferum under aerobic or anaerobic conditions.

The strains of B. fragilis subspecies fragilis and thetaiotamicron used in our study are subspecies which are common clinical isolates. These microorganisms were not found to be susceptible to the bactericidal activity of normal human serum, nor were they phagocytosed and killed intracellularly by peripheral human leukocytes in the absence of serum under anaerobic conditions. Both strains of B. fragilis were, however, phagocytosed and promptly killed intracellularly by leukocytes in the presence of normal serum. These findings suggested that these microorganisms are handled by the host in the same manner as most of the opportunist pathogens such as P. aeruginosa and E. coli. In this regard, infections caused by B. fragilis have been seen with increased frequency in the compromised host (32, 33), and the invasiveness of B. fragilis probably depends to a large extent on the functional integrity of the host's phagocytic cells and serum opsonins as well as on the virulence factors of this microorganism.

The humoral and cellular requirements for killing the strain of F. mortiferum used in our study were found to be markedly different from the requirements for killing of the B. fragilis isolates. F. mortiferum was killed directly by normal human serum in the absence of leukocytes and was also phagocytosed and rapidly killed intracellularly by leukocytes alone or by leukocytes and serum. These observations suggest that this strain of F. mortiferum is probably not invasive unless the host's humoral and cellular functions are markedly abnormal. This strain of F. mortiferum was obtained from the American Type Culture Collection and to our knowledge, was not a clinical isolate.

Our observation that gram-negative anaerobic microorganisms were killed by leukocytes in an anaerobic environment supports the findings of Mandell (34). His investigation showed that a variety of aerobic and anaerobic

microorganisms were killed efficiently by human peripheral leukocytes made anaerobic by nitrogen washout, suggesting that mechanisms other than those dependent on hydrogen peroxide may be important in intracellular killing by leukocytes. The finding that B. fragilis subspecies fragilis was killed to some extent by leukocytes in the absence of serum in the aerobic but not anaerobic environment remains to be explained but appears to be dependent upon an ingestion mechanism which occurs independently of serum opsonins.

Gram-negative non-sporulating anaerobic microorganisms constitute a major proportion of the normal flora of the human mouth, respiratory, genitourinary, and intestinal tracts. Infections caused by these microorganisms are increasingly being recognized as major problems in surgical practice (35). Gram-negative anaerobes, particularly B. fragilis, are commonly isolated in cases of secondary peritonitis in which perforation of the abdominal wall and intra-abdominal viscus permits the escape of the microorganisms into the peritoneal cavity, adjacent organs, retroperitoneal space, and circulation. Secondary peritonitis is often complicated by intra-abdominal abscesses and is most often caused by gunshot wounds of the abdomen or by other penetrating abdominal injury. This type of trauma occurs during combat in the military service and its frequency is increasing in the modern civilian population. Our studies have provided a foundation for future studies of host defense mechanisms against the microorganisms which are so commonly associated with this type of injury involving contaminated wounds.

III. EXPERIMENTAL APPROACH

Studies in three related areas of research were proposed for this renewal application. Our primary area of research continues to be the evaluation of changes in humoral components of host defense following burn trauma. This area was to be expanded to include studies of the effect of complement consumption on opsonic function in septic patients, the mechanism and significance of reduction in C3 conversion via the alternative complement pathway, and the possible production of nonspecific opsonizing antibodies following administration of P. aeruginosa vaccine. The two additional areas to be pursued were to deal with changes in the alternative and classical complement pathways in patients with other types of trauma involving contaminated wounds and in septic patients without trauma, and the role of complement and antibodies in opsonization of opportunist microorganisms which frequently cause infection in trauma patients.

Ten to 20 burned patients who are at the greatest possible risk of infection were to be selected for the complement studies. Sera were to be collected from the patients one time per week for 6 to 8 weeks postburn. The alternative and classical pathways of complement activation in the burn sera were to be assessed by measuring the levels of native C3, factor B, KAF, properdin, C3 conversion by inulin, and total hemolytic complement (CH_{50}). Blood cultures were to be drawn from the patients concurrently with serum samples and microorganisms, if present in the cultures, were to be isolated and identified. Septicemia was to be documented in the burned patients by positive blood cultures and clinical findings. If a patient developed septicemia, blood was to be drawn one or two additional times per week until the infection cleared. In those patients in which reduction in total hemolytic complement was demonstrated during septicemia, the concentration of C1, C4, C2, and C5 was to be measured in the sera to determine which early-acting classical complement components were activated. The hypothesis that microbial infection perpetuates itself in the

burned patient by causing complement consumption which reduces the opsonic capacity of the serum for the infecting microorganisms was to be tested directly. The ability of the patient's serum to promote phagocytosis and killing of his infecting microorganism by normal human peripheral leukocytes was to be tested prior to and during septicemia, and, if possible, after recovery. If more than one microorganism was isolated from a septic patient, each microorganism was to be tested separately as described above.

The frequency with which septicemia occurs in the burned patients while the C3 converting activity of their serum by inulin is reduced was also to be examined. In addition, experiments were to be performed to further substantiate that this complement abnormality was caused by a circulating inhibitor. Increasing amounts of burn sera (10%, 20%, and 50%) were to be added to 50% of pooled normal human serum, and C3 conversion by inulin was to be measured. The burn sera to be used in these experiments were to be obtained from non-septic patients. Burn sera were to be tested at times when C3 conversion by inulin was reduced and also when it was normal. Only burn sera with reduced C3 conversion should have inhibited C3 conversion in normal serum. If the appropriate burn sera reduced C3 conversion by inulin in normal serum, then the respective burn sera were to be fractionated into pseudoglobulin and euglobulin. The pseudoglobulin and euglobulin fractions of the burn sera were to be added in increasing concentrations to normal human serum, and C3 conversion by inulin was to be measured and compared to C3 conversion in normal serum supplemented with pseudoglobulin or euglobulin fractionated from pooled normal human serum. If a fraction of burn serum was shown to inhibit C3 conversion in normal serum, then preliminary studies were to be initiated to isolate the inhibitor by ion exchange and/or molecular sieve column chromatography. If inhibition of C3 conversion occurred using an unfractionated burn serum but not with its pseudoglobulin or euglobulin fractions, then both fractions of the burn serum were to be added to

normal serum and C3 conversion was to be measured, since it is possible that the inhibitory substance might be partitioned into both fractions.

For studies to determine if opsonizing antibodies for E. coli 075 were produced following administration of P. aeruginosa vaccine, antibody titers in the sera of vaccinated burned patients were to be compared to the antibody titers in the sera of non-vaccinated patients and normal volunteers. In the past, all of the burned patients at the Cincinnati General Hospital and Shriners Burn Institute received 25 μg of the vaccine upon admission, 4 and 8 days thereafter and then at weekly intervals. Recently, the policy for administration of the vaccine has changed and only patients with greater than 40% total body surface burns are given the vaccine; those patients with smaller burns are not eligible to receive it. We were to select patients with 35% to 60% total burn sizes for the study and sera from four vaccinated and four non-vaccinated patients were to be obtained weekly during 6 to 8 postburn weeks. Patients who developed septicemia were to be eliminated from the study groups. The titers of heat-stable opsonins for E. coli 075 in the sera from the burned patients and five normal volunteers were to be measured. If heat-stable opsonizing antibodies were demonstrated in the sera from either group of burned patients or from the normal volunteers, then absorption studies were to be performed to determine the specificity of the opsonizing antibodies. The titers of heat-stable opsonins for E. coli 075 were to be determined in several of the burn and/or normal sera after absorption with the homologous E. coli strain and with P. aeruginosa. If the heat-stable opsonizing activity for E. coli 075 was removed following absorption of the sera with the E. coli and P. aeruginosa strains, then strains of S. marcescens, P. mirabilis, and B. fragilis were to be used for absorption to further examine the specificity of the antibodies.

Another new area of research which was planned for the investigation was a preliminary study to assess the alternative and classical complement pathways

in patients with non-burn trauma and in septic patients without trauma. Ten patients with severe blunt or penetrating abdominal trauma and five to ten septic patients without diseases associated with complement abnormalities were to be studied. Sera were to be obtained within 72 hours following the injury from the trauma patients. The concentrations of native C3, factor B, properdin and KAF, conversion of C3 by inulin and total hemolytic complement in the sera from both groups of patients were to be measured.

Studies to define which components of alternative complement pathway participate in opsonization of E. coli 075 and the clinical isolate of P. aeruginosa were to be delayed until factor $\overline{D}$ was obtained from normal human serum in purified form and until the bacterial strains had been demonstrated to activate the alternative complement pathway in normal human serum. Two different methods were to be used to purify factor $\overline{D}$. The protein preparations were to be compared functionally by testing their ability to convert purified C3 in the presence of purified factor B. Additional reasons for purifying human factor $\overline{D}$ and for preparing antisera to this protein were for the future measurement of this critical alternative pathway component in the sera from trauma patients and for performing restoration experiments described in the section on the role of complement and antibody in opsonization of B. fragilis. To determine if the strains of E. coli and P. aeruginosa activated the alternative complement pathway, the ability of washed cells to convert C3 in pooled normal human serum chelated with ethylene glycol tetra-acetic acid (EGTA) supplemented with $MgCl_2$ was to be tested. Unlike EDTA, which binds both Ca^{++} and Mg^{++}, EGTA has been shown to chelate primarily Ca^{++}, which has not been found to be a required cofactor for alternative pathway function. Conversion of C3 in EGTA-treated serum supplemented with Mg^{++} would indicate that the bacterial strain could activate the alternative pathway. The strains were also

to be tested for their ability to promote lysis of glutathione-treated erythrocytes in the presence of normal human serum, and to consume C3 through C9 in C4-deficient guinea pig serum; both of these functions are also mediated by the alternative pathway.

Extensive experiments were to be performed to define the role of antibody and complement in opsonization of B. fragilis subspecies thetaiotaomicron and fragilis. The ability of normal human serum depleted of complement or antibody to promote phagocytosis and intracellular killing of the bacterial strains by human peripheral leukocytes was to be tested under anaerobic conditions and compared to untreated normal serum. In attempting to assess the role of complement in opsonization, standard anticomplementary procedures (heating at 56°C for 30 minutes) was to be used. To determine if antibody was required for opsonization of the bacterial strains, normal human sera absorbed at 0°C with washed cells of the homologous or heterologous strain of B. fragilis were to be tested for their opsonic activity against both strains. This approach should provide information regarding the specificity of human antibodies to B. fragilis. If a requirement for antibody for opsonization of the bacterial strains was demonstrated, then antibody depleted serum was to be supplemented with normal IgG prior to retesting.

If complement was shown to be required for opsonization of the bacterial strains, then studies were to be initiated to determine the mechanisms of complement activation which were involved and to determine the participation of late-acting complement components. For these experiments, use was to be made of sera from animals and humans with known, genetic deficiencies of complement components C4, C6 and C8. Comparison of the opsonic activities of normal and deficient sera were to be made. The ability of the bacterial strains to be phagocytosed and killed in the presence of normal human sera depleted of properdin, factor B, or factor $\overline{D}$ of the alternative complement pathway was

also to be tested. If opsonic activity of one of these sera was found to be reduced then restoration of opsonic activity of the serum was to be attempted using the respective partially purified protein.

IV. PROGRESS REPORT

A. Changes in Humoral Components of Host Defense in Patients Following Burn Injury

1. Studies to determine the association between changes in serum factors and septicemia in burned patients

a. Results

In the previous preliminary studies presented in our Annual Summary Report (June, 1976), reduction in the immunochemical levels and functional activities of components of the classical and alternative complement pathways was associated with septicemia in two burned patients (9). Reduction in the classical complement pathway occurred during the first 10 days postburn in an infant who developed septicemia with S. aureus on the third postburn day which persisted through the sixth day. Reduction in both the classical and alternative complement pathways was also observed in a 39 year old female who had multiple episodes of Pseudomonas septicemia and candidemia during her clinical course and who died of septic shock. The present investigation was undertaken to determine if these preliminary data could be substantiated and to determine if complement consumption reduced the opsonic capacity of the patient's serum for his own infecting microorganism.

Fifteen patients with severe thermal injury were followed for up to 9 weeks postburn. Patients who appeared to be at the greatest possible risk of infection because of burn size or age or both were selected for the study. Serum samples were obtained from the patients as soon after the injury as possible and then at weekly intervals. In those patients who developed septicemia, serum samples were also obtained two additional times per week until blood cultures became negative. Blood cultures were drawn on all patients at least one time per week by our staff and additional blood cultures were drawn at the discretion of the of the attending physicians. This procedure was adopted for the purpose of

documenting negative as well as positive cultures obtained on the patients. Septicemia was documented by clinical findings and positive blood cultures. The clinical criteria used for the diagnosis of septicemia were (a) chills and fever, (b) tachycardia, (c) hypotension, and (d) disorientation.

Six of the fifteen patients had one or more positive blood cultures during their clinical course. Clinical characteristics of this group of patients including information regarding blood cultures are given in Table 1. The other nine patients had negative blood cultures. Clinical characteristics and grouping of these burned patients are shown in Table 2.

In all three groups of patients, levels of native C3, KAF, and factor B were normal or elevated for the entire study period (Fig. 2). Properdin levels were initially reduced in all groups. Properdin levels remained reduced in patients with the largest burn sizes (group A) for 40 days postburn and in patients with intermediate burn sizes (group B) for 30 days postburn. In patients with the smallest burn sizes (group C), properdin levels were normalized during the sixth to tenth postburn day period. Conversion of C3 by inulin was markedly reduced in group A patients for the duration of the study. In group B patients, C3 conversion by inulin was normal during the first 40 days postburn and then was subsequently reduced. In group C patients, C3 conversion was normal for the entire study period. Total hemolytic complement (CH_{50}) was normal in all three groups for the duration of the study.

The first patient (Patient I) in the group of infected patients had one positive blood culture on day 4 with P. mirabilis which was unassociated with fever or any other clinical findings associated with septicemia. A change in the complement profile in this patient was not associated with the transient bacteremia, and opsonic activity of sera from this patient for his infecting strain of P. mirabilis was normal during his entire clinical course of 30 days postburn (Fig. 3). C3 conversion by inulin and properdin concentration

Table I. Clinical Characteristics of the Septic Burned Patients

Patient No.	Age	Sex[a]	Body Surface Injured[b]		Infecting Microorganisms	Blood Cultures[c]	
			Total %	Third Degree %		Positive Day	Negative Day
I	51	M	35	13	P. mirabilis	4	13, 21
II	3	M	45	45	S. aureus C. albicans	7, 11, 20 30, 32	13, 25, 29, 35, 42
III	13	F	80	53	S. aureus S. epidermidis	13, 20, 48, 49 27, 43, 48	6, 14, 17-19, 24-26, 28, 31-35, 38, 39, 44, 46, 57, 58
IV	12	M	70	60	S. aureus S. albicans S. faecalis	9-13, 34 22 27	15, 18-20, 28-31, 36-40, 42, 45, 46, 48
V	59	F	63	13	S. aureus	4, 6, 11	-
VI	3	F	82	82	S. aureus E. coli	17, 19 19, 21	3, 10, 22

[a] M = male; F = female.

[b] All patients had flame burn injuries.

[c] Numbers indicate the number of days following the injury that positive or negative blood cultures were obtained.

Table 2. Clinical Characteristics of the Non-Septic Burned Patients

Group	Patient No.	Age	Sex[a]	Body Surface Injured[b]		Average Burn Ratio per Group
				Total %	Third Degree %	
	1	5	M	75	70	
A	2	10	M	71	66	68/65
	3	6	F	60	60	
	1	5	F	54	43	
B	2	42	M	55	2	52/20
	3	9	F	49	15	
	1	12	F	40	11	
C	2	10	M	37	16	35/11
	3	49	M	30	6	

[a] M = male; F = female.

[b] All patients had flame burn injuries.

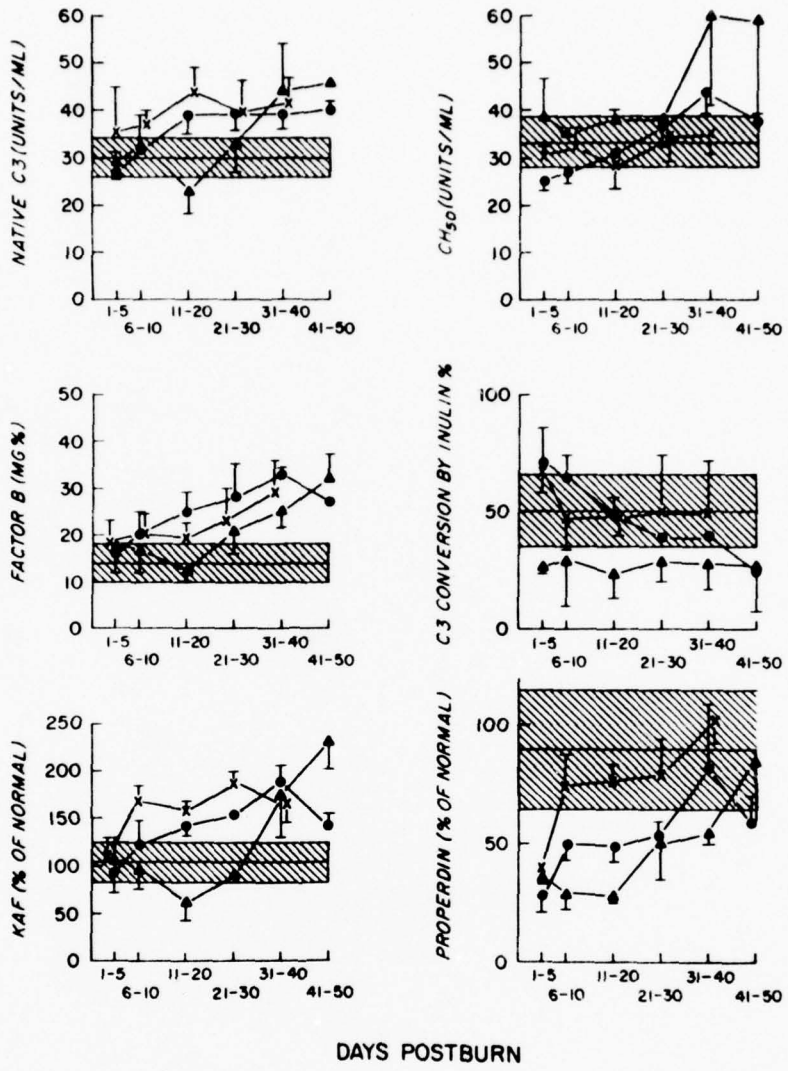


Fig. 2. Immunochemical levels and functional activities of components of the classical and alternative complement pathways in the sera of nine non-septic burned patients. The average burn ratio (total burn size (%)/ area of third degree burn (%)) was 68/65 for group A, 52/20 for group B, and 35/11 for group C. Refer to Table 2 for data on individual patients in each group. The solid triangles represent mean values for group A patients, the solid circles represent mean values for group B patients, the solid circles represent mean values for group B patients, and the crosses represent mean values for group C patients. The shaded areas represent the variation of 20 normal sera (mean $\pm$ 1 SD), and the vertical bars represent the standard error of the mean.

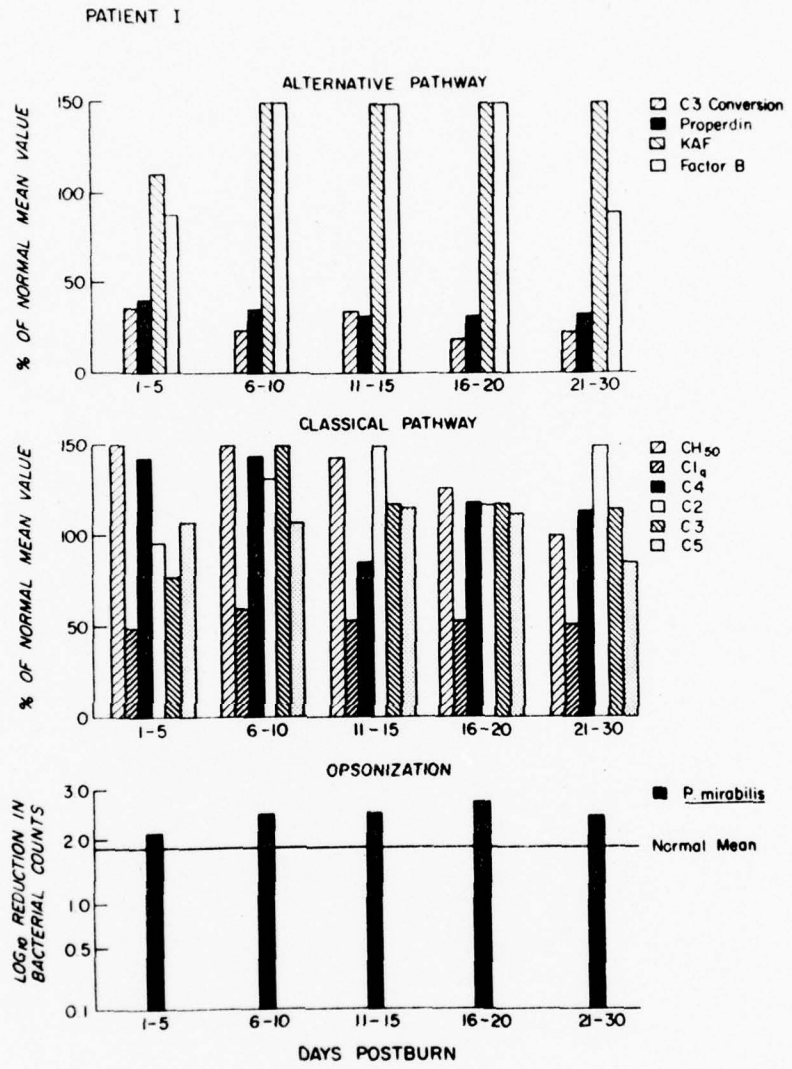


Fig. 3. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of the patient's infecting strain of P. mirabilis in the sera from Patient I. Clinical information on this patient is given in Table 1.

were reduced in this patient during the entire postburn period; levels of factor B and KAF were normal or elevated. Functional activity of the classical pathway as measured by total hemolytic complement (CH_{50}) was normal or elevated, although the level of Clq in the patient's sera was approximately 50% of normal for the entire study period.

Patient II had multiple positive blood cultures for S. aureus during the 7th to 20th postburn days and for C. albicans on the 30th and 32nd postburn days. The blood culture results indicated that this patient was culture negative during the 21st through the 29th postburn day and after the 32nd postburn day. This patient was febrile with temperatures ranging from 101° to 103° during the entire study period of 40 postburn days, but had no other clinical findings associated with septicemia. Reduction in the classical complement pathway was found to be associated with the septic episodes (Fig.4). CH_{50} and immunochemical levels of C1 and C4 were reduced during the first ten-day postburn period and during the 31st to 40th postburn period. Reduction in these measurements was not, however, demonstrated during the 11th to 20th or 21st to 30th postburn day periods, although the patient had positive cultures for S. aureus on the 11th and 20th postburn days. Reduction in C3 conversion by inulin and properdin concentration in this patient's sera could not be related to septicemia. No significant reduction in opsonic activity of the patient's sera for his infecting strain of S. aureus was demonstrated during the entire study period. The ability of the patient's sera to opsonize his infecting strain of C. albicans was unable to be evaluated, since pooled normal serum at a concentration as high as 20% was unable to promote phagocytosis and killing of this microorganism by normal leukocytes.

Patient III had multiple positive blood cultures for S. aureus and S. epidermidis during the 13th to 27th and 43rd to 49th postburn day periods.

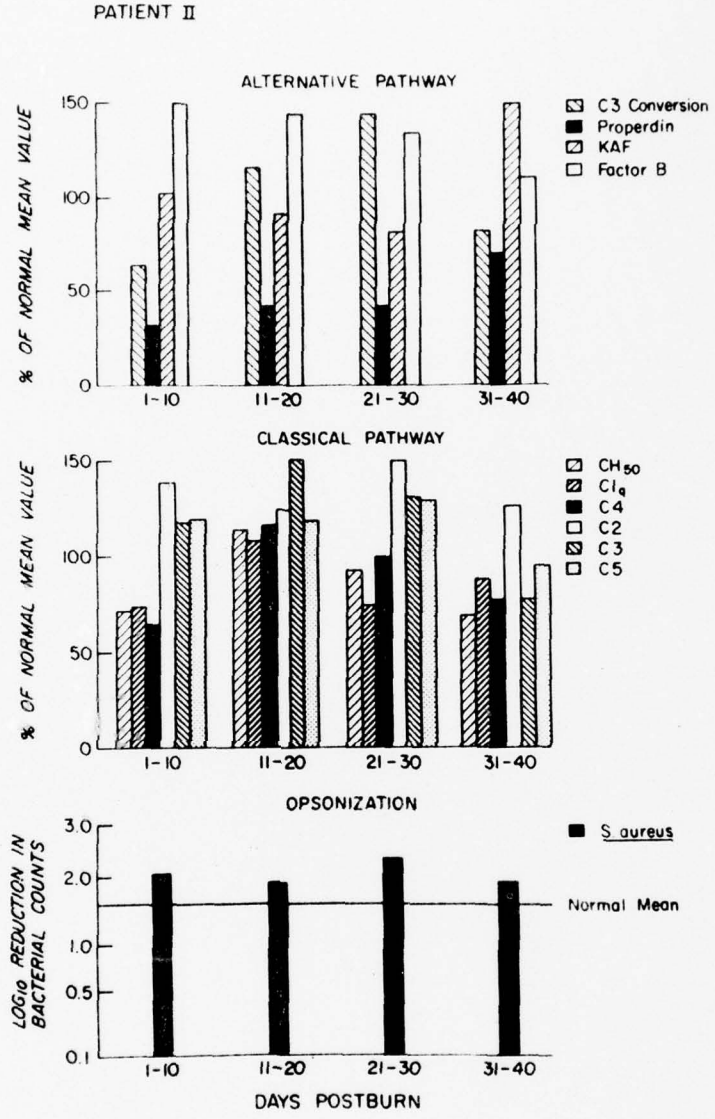


Fig. 4. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of patient's infecting strain of S. aureus in the sera from Patient II. Clinical information on this patient is given in Table I.

This patient also had negative blood cultures during these time intervals. In addition, negative cultures were obtained prior to the 13th, during the 28th to 43rd postburn days, and after the 49th postburn day. This patient was febrile with temperatures ranging from 100° to 103° during the entire study period and had a consistent pulse rate of 140 to 160 during this time. The patient had no documented episodes of hypotension or disorientation. Reduction in CH_{50}, levels of C1, C4, C5, and to a lesser extent levels of C2 and C3 was demonstrated during the first 25 postburn days, but not during the second septic period (43rd to 49th days) (Fig.5). Alternative pathway activation followed the normal pattern which has been observed in a patient with this burn size. C3 conversion was reduced after the 6th to 15th postburn day period, the level of properdin was reduced and levels of factor B and KAF were normal or elevated for the duration of the study. No decrease in the ability of the patient's sera to opsonize her infecting strains of S. aureus was demonstrated. Reduction in the opsonic activity of the patient's sera for her infecting strain of S. epidermidis was only demonstrated during the first five days postburn.

Patient IV was admitted to the Shriners Burn Institute nine days following his burn injury and had positive blood cultures with S. aureus during the 9th to 13th postburn day period. Blood cultures were negative thereafter until the 22nd and 27th postburn days when blood cultures were positive for C. albicans and S. faecalis respectively. Blood cultures were negative from the 28th to the 31st postburn day period with the last positive culture being obtained on the 34th postburn day for S. aureus. The patient was febrile with temperatures ranging from 101° to 104° for the entire study period. No other clinical findings associated with septicemia were observed in this patient. Consumption of components of the classical complement pathway was observed during the first

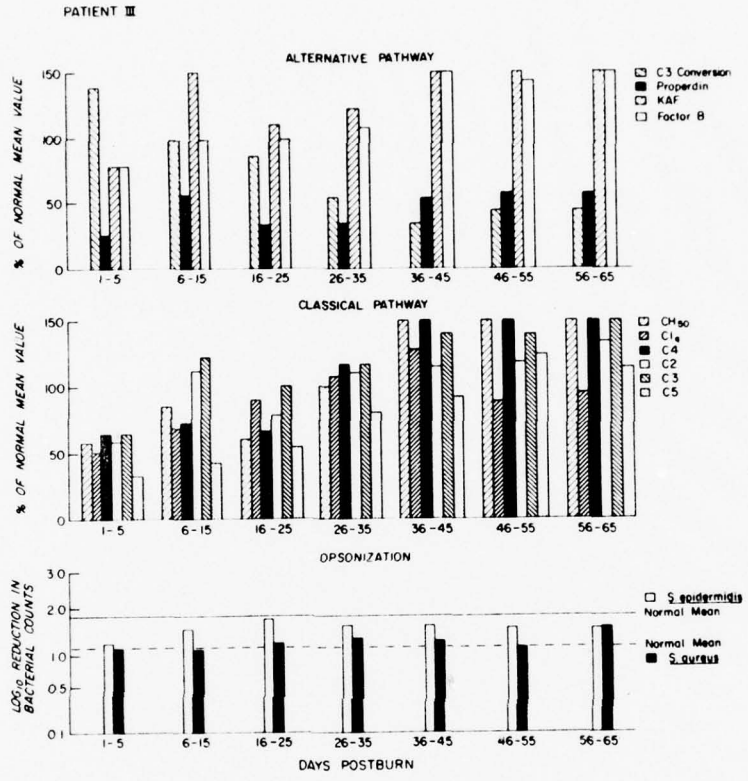


Fig. 5. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of the patient's infecting strain of S. epidermidis and S. aureus in the sera from Patient III. Clinical information on this patient is given in Table I.

35 postburn days (Fig.6). CH_{50}, levels of C1, C4, C2, and C5 were more markedly decreased initially during the 9th to 15th postburn day period; consumption was not, however, extensive enough to decrease the C3 concentration. Alternative pathway activation followed the normal pattern which has been observed for a patient with this burn size as described for Patient III. In addition, no impressive decrease in the ability of the patient's sera to opsonize his infecting strain of S. aureus or Streptococcus faecalis was demonstrated. The opsonic activity of the patient's sera for his infecting strain of C. albicans was unable to be evaluated, since pooled normal human serum at a concentration as high as 20% was unable to promote phagocytosis and killing of this microorganism by normal leukocytes. This finding is identical to the results obtained with the C. albicans strain which was isolated from Patient II.

Patient V had multiple positive blood cultures with S. aureus during the 4th to 11th days postburn and died of what appeared to be septic shock on day 12. She was febrile during the entire study period with temperatures ranging from 101° to 102°, became disorientated on day 8 and was responsive only to deep pain by day 12. She had no documented episodes of tachycardia; blood pressure measurements were unable to be performed due to the extent of the patient's injury. Consumption of the classical complement pathways was marked during the entire ten day postburn period; alternative pathway consumption was observed only during the first three days postburn (Fig.7). C3 conversion by inulin, CH_{50}, levels of C1, C4, C2, C5 and to a lesser extent C3 were decreased during the ten day period, and factor B levels were decreased during the first six days postburn. No marked decrease in opsonic activity of the patient's serum for her infecting strain of S. aureus was demonstrated during the entire study period.

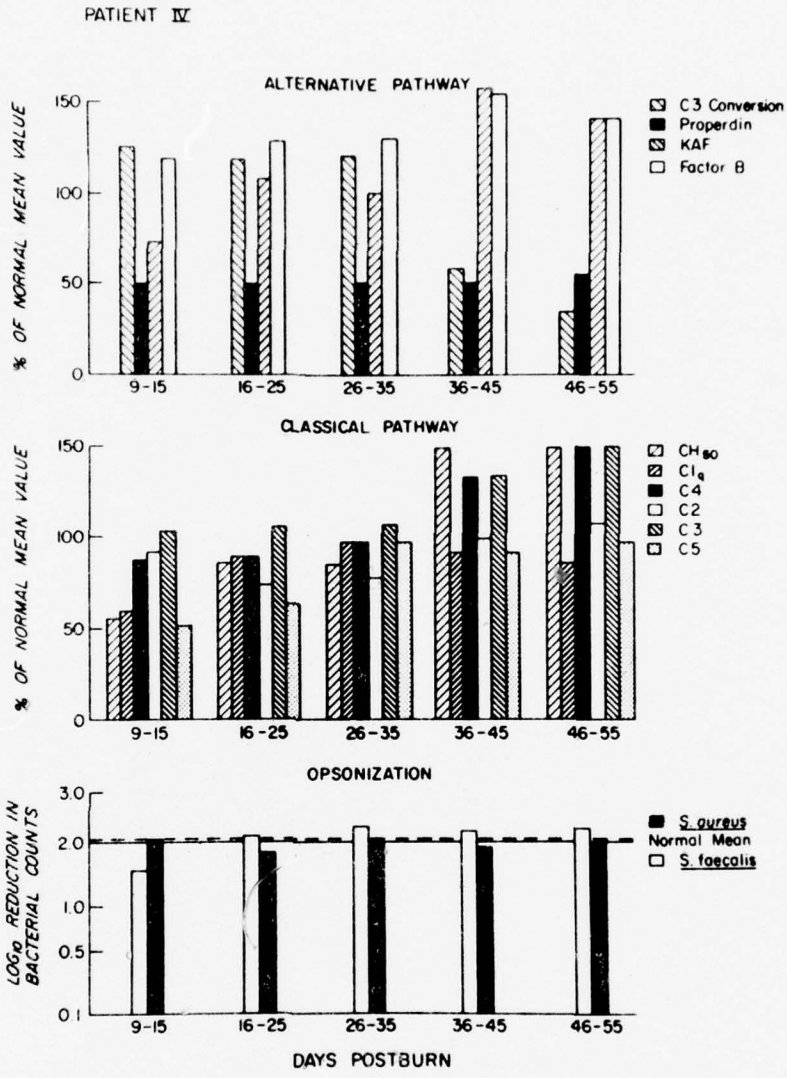


Fig. 6. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of the patient's infecting strain of S. aureus and S. faecalis in the sera from Patient IV. Clinical information on this patient is given in Table 1.

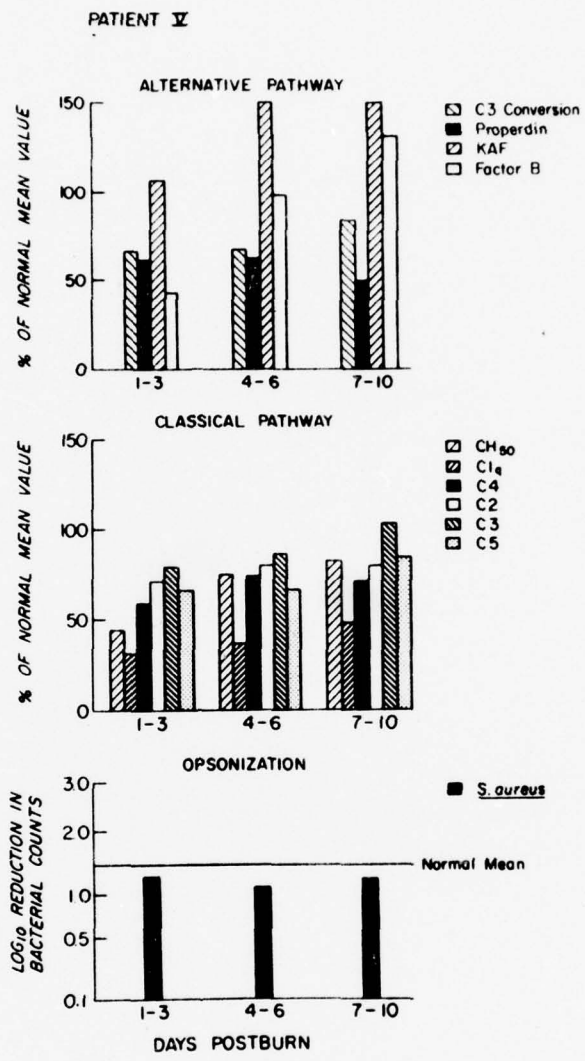


Fig. 7. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of the patient's infecting strain of S. aureus in the sera from Patient V. Clinical information on this patient is given in Table 1.

Patient VI had multiple positive blood cultures with S. aureus and E. coli during the 17th to 21st postburn days and died of what appeared to be septic shock on day 27. She was febrile with temperatures ranging from 101° to 102° during her entire clinical course. She had intermittent episodes of tachychardia on days 2, 3, 11, 12, 15, 20, 24 to 27. She was hypotensive upon admission (day 2) and on days 19, 20, 26, and 27. She was alert through the 11th postburn day. Classical pathway activation was markedly reduced during the entire study period of 25 postburn days (Fig.8). Functional activity of her alternative pathway as assessed by C3 conversion by inulin was unusually normal, and the only abnormality which was noted was decreased KAF levels. Since KAF is a regulatory protein of the classical as well as the alternative complement pathway, this protein was probably consumed along with other components of the classical pathway.

Opsonization of the patient's infecting strain of S. aureus by the patient's sera was not substantially affected by the decreased classical pathway activity. However, the ability of the patient's serum to opsonize her infecting strain of E. coli was markedly reduced during the first five days postburn and then again during the 21st to 25th postburn day period.

The types and amounts of blood products administered to the septic patients is shown in Fig. 9. A wide variation in the regimen for administration of blood products was observed. Patients I, II, V, and VI received the least amount of blood products; patient I only received packed cells during the first five days postburn and then no blood products thereafter. Patients III and IV received from 500 to 3600 ml of whole blood and from 400 to 3900 ml of single donor plasma per ten day period.

b. Discussion

The data presented in this section although preliminary,

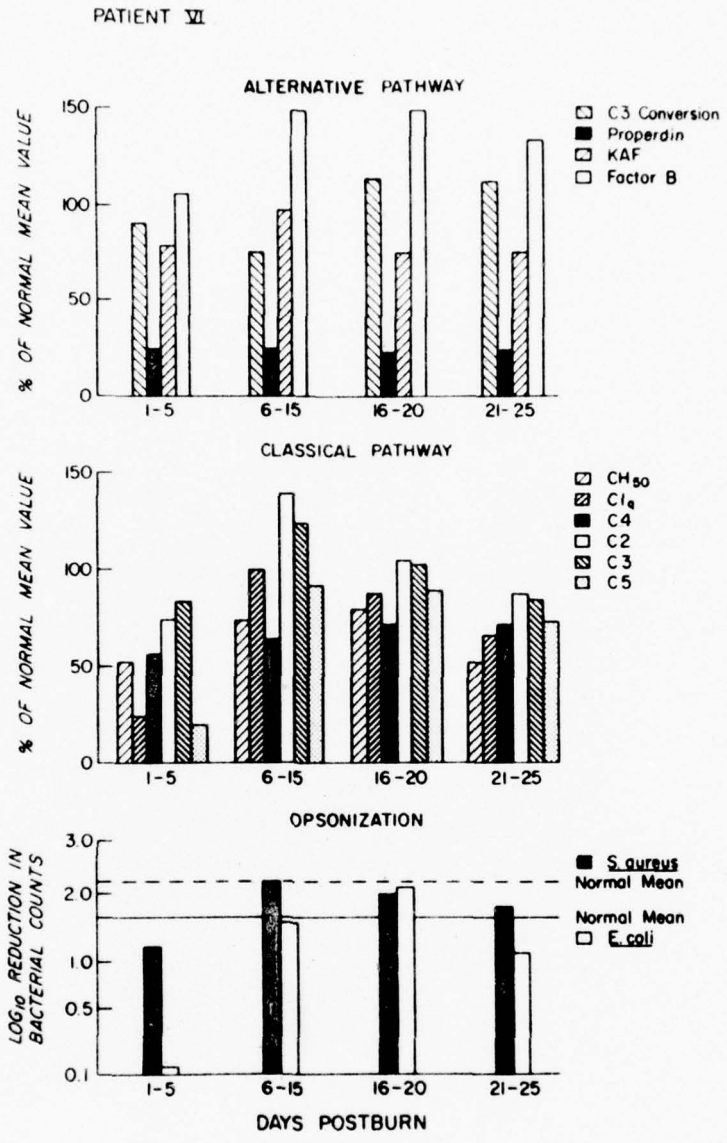


Fig. 8. Immunochemical levels and functional activities of components of the classical and alternative complement pathways, and opsonization of the patient's infecting strains of S. aureus and E. coli in the sera from Patient VI. Clinical information on this patient is given in Table 1.

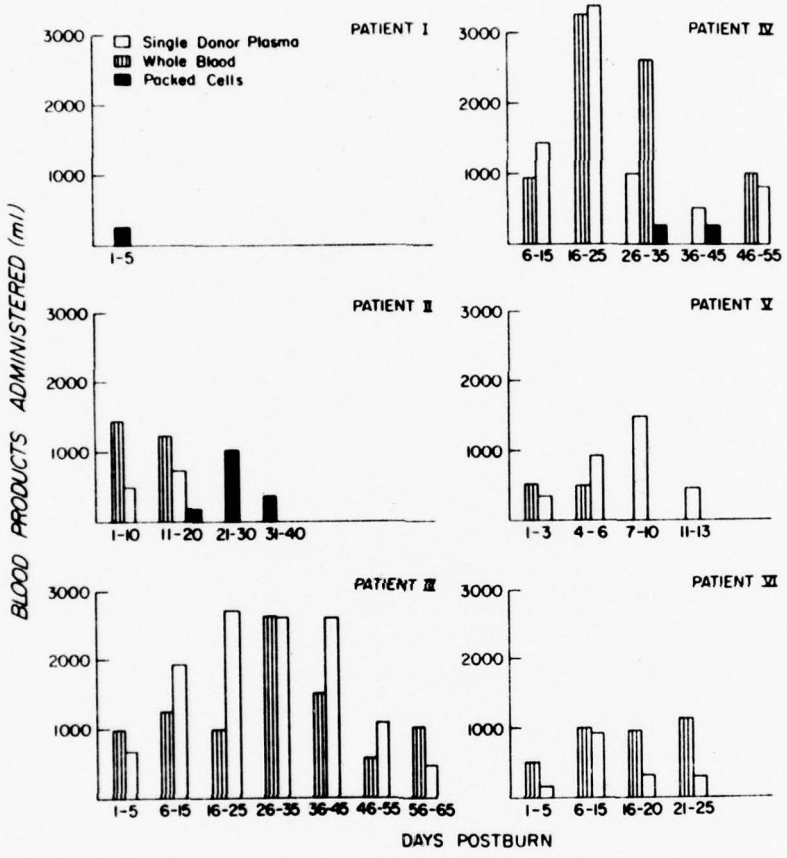


Fig. 9. Temporal sequence of administration of blood products to the burned patients with positive blood cultures.

indicate that consumption of the classical complement pathway was associated with and was probably caused by septicemia in thermally injured patients. In three of five burned patients (Patients IV, V, and VI), decrease in the functional activity and immunochemical levels of components of the classical complement pathway occurred prior to and during septic episodes. Both of the other two patients also had decreased classical pathway activity prior to the development septicemia. In one of the patients (Patient III), classical pathway activity was decreased during the first septic episode (13th to 27th postburn days) but not the second (43rd to 49th postburn days). In the other patient (Patient II), classical pathway activity was decreased during the second septic period (30th to 32nd postburn days) but not for the duration of the first episode (11th to 20th postburn days). The results obtained in Patient II can probably be attributed to the administration of blood products, since this patient received whole blood and single donor plasma during the first but not the second septic episode. Patient III, whose classical pathway activity was reduced during the first but not the second septic episode, received equivalent amounts of single donor plasma per day during the two time intervals but twice the amount of whole blood per day during the second septic episode. However, Patient IV showed classical pathway consumption during his septic episodes, when he was receiving as much whole blood and single donor plasma as Patient III. It should be mentioned that the lack of consistency in the changes in complement observed in these two patients might be related to differences in the complement levels in the blood products which they received. We have measured complement levels and activities in three different units of single donor plasma and found them to vary markedly; one of the units contained 25% of the normal amount of classical and alternative pathway activity.

One of the patients (Patient I) in our study, who was bacteremic, served as an excellent control for determining the relationship between changes in complement and septicemia. No changes in the classical or alternative complement pathways occurred prior to or on the day that this patient's blood culture was positive. An early reduction in classical pathway activity was demonstrated in all of the septic patients during the initial postburn period, suggesting that this humoral abnormality may be predictive of a septic episode and may possibly predispose the patient to infection. Our results tend to rule out the possibility that the intial reduction in classical pathway activation occurring during the first week postburn results from infection, since blood cultures were consistently negative in all but one of the patients during this time.

The classical pathway appeared to be activated preferentially in the burned patients during septic episodes. Although C3 conversion by inulin was often reduced during septic episodes, levels of factor B were generally normal or elevated. Since it is well known that factor B is consumed during alternative pathway activation, the results suggest that C3 conversion via the alternative pathway was reduced in the burned patients due to inhibition rather than to consumption of alternative pathway components. Inhibition of alternative pathway activation would provide an explanation for the observed preferential activation of the classical pathway. Further studies regarding evidence for presence of a circulating inhibitor of C3 conversion via the alternative pathway in burned patients is presented in the next section of this report. This topic is also discussed in section C1 of this report dealing with preferential utilization of the classical pathway in burn sera during opsonization of E. coli 075.

In only one patient did consumption of components of the classical

complement pathway occurring during septicemia decrease the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of E. coli; sera from the same patient which could not opsonize E. coli, opsonized her infecting strain of S. aureus normally. The microorganisms which were isolated from the other septic burned patients and used to test the opsonic capacity of the patients' sera were also, with one exception, strains of Staphylococci. Since there is evidence that S. aureus can be opsonized by normal IgG in the absence of complement (31, 36), the lack of demonstration of reduction in the opsonic capacity of the patients' sera for their infecting strains of Staphylococci might be related to the lack of requirement for complement for opsonization of the strains. It will be very important in future studies to determine if reduction in serum opsonic activity occurs for certain bacteria and not for others as was observed in this group of septic patients.

It should be emphasized that the E. coli isolate as well as the strains of Staphylococci and S. faecalis isolated from the burned patients were not susceptible to direct lysis by pooled normal serum in the absence of leukocytes or to phagocytosis and intracellular killing by normal leukocytes in the absence of serum. In addition, the concentration of serum and incubation periods used in the opsonic assays were specific for each infecting microorganism. Concentrations of the patients' sera were based on the minimal amount of pooled normal human serum which was found to promote maximal intracellular killing of the microorganism by normal leukocytes during the shortest incubation period.

Another interesting observation which was derived from this study was that strains of C. albicans isolated from the burned patients were not phagocytosed and killed intracellularly by normal leukocytes in the presence of

five to ten times the concentration of normal human serum required for phagocytosis of other microorganisms. Further studies will be initiated to determine if the C. albicans strains isolated from the burned patients are more resistant to opsonization by normal serum than strains of C. albicans isolated from other sources and to determine the human serum proteins required for opsonization of the C. albicans strains.

Data from the burned patients who did not develop septicemia was grouped according to burn size to enable comparisons to be made between the present study group and previously studied patients (9). The only difference which was observed in the data from the groups of burned patients in this study and our previously studied groups of patients with similar burn sizes was in the results of the analyses of C3 conversion by inulin in patients with the largest burn sizes. In the present study, C3 conversion by inulin was markedly reduced initially and remained reduced for the duration of the study. In the previous study, C3 conversion was not reduced until after the first ten days postburn. However, in our original work demonstrating reduction in C3 conversion in the sera of patients with severe thermal injury, C3 conversion in the sera of some of the patients was reduced during the initial ten day postburn period and in others it was not (8). The discrepancy in the C3 conversion results remains to be explained, however it does not appear to be related to burn size.

2. Studies to determine the mechanism of reduction in C3 conversion via the alternative pathway in burned patients

a. Results

Our previous studies showed that conversion of C3 by inulin in sera from severely burned patients was reduced after the first 10 days postburn and was normalized by the seventh week (8, 9). The occurrence and duration of the reduction in C3 conversion were found to be directly related to the severity of the burn injury. Reduction in the converting activity of the burn sera could not be fully restored to normal by addition of 50% pooled normal human serum (PNHS), providing preliminary evidence that this complement abnormality was caused by an inhibitor. The present investigation was undertaken to test directly the hypothesis that reduction in C3 conversion by inulin in the burn sera was caused by a circulating inhibitor.

C3 conversion by inulin was measured during 6 to 8 weeks postburn in the sera of seven patients with burn injuries ranging in size from 37% to 92%. Pertinent clinical information on the patients is given in Table 3. In all of the patients, C3 conversion was reduced after the tenth postburn day (Fig. 10). With the exception of patient 3, restoration of the C3 converting activity in the patients' sera occurred during the 5th to 6th week postburn. In patient 3, C3 conversion remained reduced during 59 postburn days; the duration of the reduction appeared to be related to the type of burn injury.

Increasing amounts of burn or normal sera (10 ul, 20 ul, and 50 ul) were added to 50 ul of PNHS. The volumes were adjusted to 100 ul with saline, and C3 conversion by 10 mg/ml of inulin was measured. Addition of normal sera obtained from individual donors to PNHS did not reduce C3 conversion by inulin (Fig.11). Addition of normal serum depleted of complement components required for C3 conversion (PNHS heated at 56°C for 30 minutes) to PNHS resulted in a slight

Table 3. Clinical Characteristics of Patients with Burn Injury

Patient No.	Age[a]	Sex[b]	Cause of Burn	Body Surface Injured Total %	Third Degree %
1	2	F	immersion scald	37	30
2	2	M	flame	45	15
3	16	F	sulfuric acid contact	60	50
4	21	M	flame	73	56
5	5	M	flame	73	70
6	59	M	flame	45	27
7	30	M	flame	92	0

[a] Patients ranging in age from 2 to 11 years were hospitalized at the Shriners Burn Institute and patients over 15 years of age were hospitalized at the Cincinnati General Hospital.

[b] M = male; F = female.

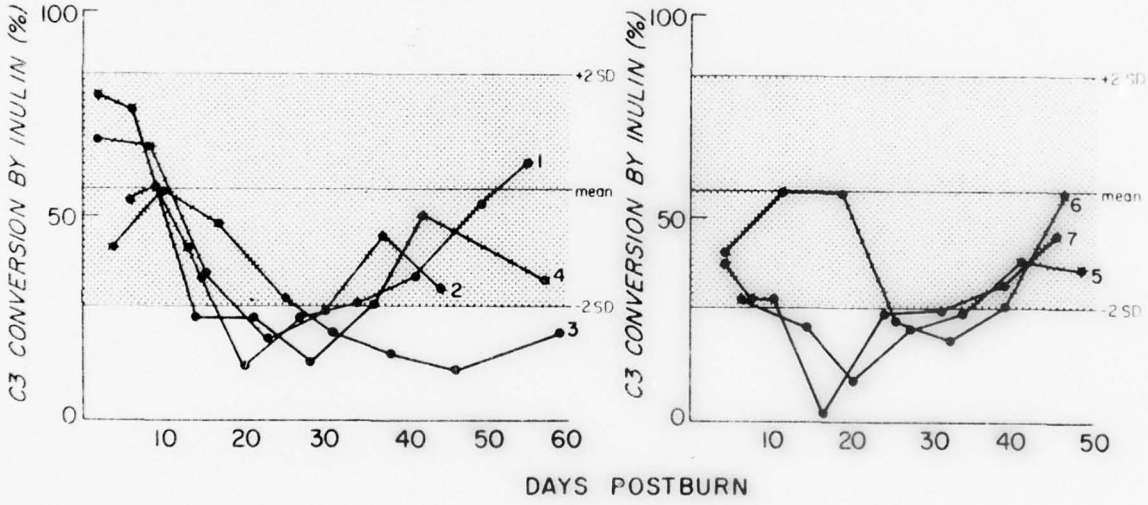


Fig. 10. C3 conversion by inulin in the sera from patients 1 to 7 during 60 days postburn. The numbers following each line correspond to patient numbers. The shaded area represents the mean ± 2SD of C3 conversion by inulin in 15 normal sera.

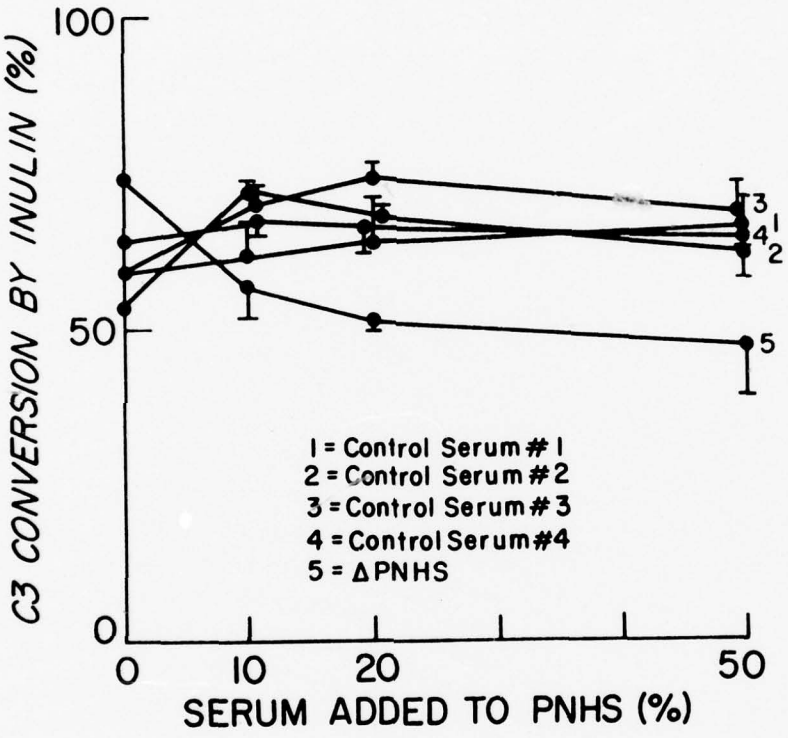


Fig. 11. C3 conversion by inulin in PNHS supplemented with individual normal control sera or heated PNHS (56°C, 30 minutes). C3 conversion by inulin in the unsupplemented control sera was as follows: (1) 46%, (2) 65%, (3) 54%, (4) 49%, (5) 0%. The points represent mean values of 2 to 4 determinations, and the vertical bars represent the standard error of the mean.

reduction in C3 conversion, although the values did not differ significantly from those obtained when untreated normal sera were added to PNHS. The complement depleted control was included for the purpose of showing the extent of C3 conversion achieved when PNHS and a serum deficient in complement components were added together. If reduction in C3 conversion by inulin in a burn serum resulted from a deficiency of critical proteins required for C3 conversion, then values of approximately 48% $\pm$ 8% (mean $\pm$ s.e.m.) or greater should be obtained at a 50% serum concentration. Any further reduction in C3 conversion upon mixture of equal parts of burn serum and PNHS should result from an inhibitory substance rather than from a deficiency of serum proteins.

Sera obtained from patients 1 to 5 were added in increasing concentrations (10 ul, 20 ul, and 50 ul) to 50 ul of PNHS. The volumes were adjusted to 100 ul with saline, and C3 conversion by 10 mg/ml of inulin was measured. Burn sera were tested at times when C3 conversion was reduced and also when it was normal. None of the burn sera with normal C3 conversion inhibited C3 conversion to any extent when added to PNHS (Fig.12). The C3 conversion values of the burn sera prior to their addition to PNHS and the days the sera were collected are given in the figure. Sera from patients 2, 3, and 4 with reduced C3 conversion inhibited C3 conversion when added to PNHS. Two of the three sera with reduced C3 conversion from patient 5 also inhibited C3 conversion in PNHS. The third serum obtained on day 20 from this patient had reduced C3 conversion, but did not inhibit C3 conversion when added to PNHS. In addition, serum from patient 1 with reduced C3 conversion did not inhibit C3 conversion in PNHS. When the data were subjected to statistical analysis using the Student t test, the differences which were observed between the burn sera with reduced C3 conversion and those with normal C3 conversion were not found to be significant by the Student t test at a p value of less than 0.05. In addition, the values obtained

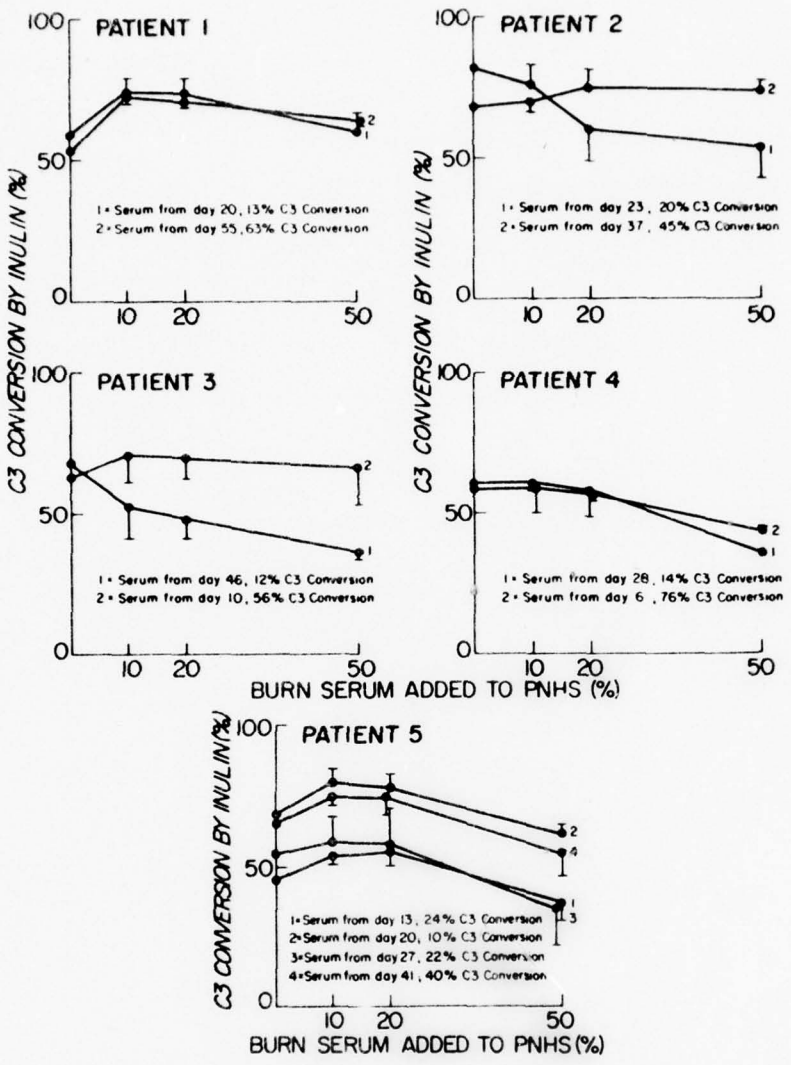


Fig. 12. C3 conversion by inulin in PNHS supplemented with increasing concentrations of patients' sera. The values for C3 conversion in the sera from each patient prior to their addition to PNHS and the days postburn that the sera were collected are given in the figure. The points represent mean values of 2 to 4 determinations, and the vertical bars represent the standard error of the mean.

when equal parts of burn serum and PNHS were added together were not considerably lower than those obtained when the complement deficient normal serum was added to PNHS (refer to Fig.11).

One possible explanation for the lack of statistically significant differences between burn sera with reduced versus normal C3 conversion might be that the inhibitor of C3 conversion was present in a low concentration in the burn sera. An alternative hypothesis was that reduction in C3 conversion by inulin in the burn sera was caused by a deficiency of critical normal serum proteins required for C3 conversion. To test the former hypothesis, burn sera were fractionated into pseudoglobulin and euglobulin with the hope that the inhibitor, if present in the sera, might be partitioned into one of these fractions and could thereby be concentrated. Sera (2 ml) from patients 3,4, and 5 with reduced C3 conversion which had been found to inhibit C3 conversion when added to PNHS (refer to Fig.12) were dialyzed against 0.008M EDTA, pH 5.4 for 18 to 24 hours at 4°C. The euglobulin from each serum was deposited by centrifugation, and the pseudoglobulin was removed. The euglobulin was washed three times with 0.008M EDTA and redissolved in 0.5 ml of 0.01M Tris-HCl, pH 7.4, containing 0.3M NaCL. The euglobulin and pseudoglobulin fractions of the sera were then dialyzed against 0.01M phosphate buffered saline containing 5×10^{-4}M $CaCl_2$ and 1.5×10^{-4}M $MgCl_2$. PNHS (2 ml) were fractionated into pseudoglobulin and euglobulin fractions and dialyzed as described above for fractionation of the burn sera.

Unfractionated burn sera and the pseudoglobulin and euglobulin fractions of the burn sera were added in increasing concentrations to PNHS, and C3 conversion by 10 mg/ml of inulin was measured and compared to C3 conversion in PNHS supplemented with pseudoglobulin or euglobulin fractionated from PNHS. The euglobulin fractions of the burn sera were found to markedly inhibit C3

conversion by inulin (Fig.13). The euglobulin prepared from PNHS was also found to inhibit C3 conversion when added to PNHS. The difference between inhibition by normal versus burn euglobulin was not found to be statistically significant by the Student t test at a p value of less than 0.05, however the burn euglobulin was definitely more inhibitory than the normal euglobulin. Pseudoglobulin prepared from either normal or burn sera enhanced C3 conversion by inulin when added to PNHS. These preliminary results suggested that a euglobulin factor or factors which regulate C3 conversion in normal serum might be present in an elevated concentration in the burn sera causing inhibition of C3 conversion.

Sera from patients 5, 6, and 7 with reduced C3 conversion were pooled, and each pooled serum was fractionated into pseudoglobulin and euglobulin as described above. Unfractionated pooled sera and the pseudoglobulin and euglobulin fractions of the pooled sera were added in increasing concentrations to PNHS, and C3 conversion by 10 mg/ml of inulin was measured and compared to C3 conversion in PNHS supplemented with pseudoglobulin or euglobulin fractionated from PNHS. The euglobulin fractions of the pooled burn sera inhibited C3 conversion by inulin in PNHS to a greater extent than the euglobulin fractionated from PNHS (Fig.14). Pseudoglobulin prepared from either burn sera or PNHS enhanced C3 conversion when added to PNHS. These results confirmed our previous data obtained using individual rather than pooled burn sera. The only difference in the results was that the unfractionated pooled burn sera did not inhibit C3 conversion in PNHS to as great an extent as the unfractionated individual burn sera.

The euglobulin prepared from the pooled burn sera from patients 6 and 7 and the euglobulin prepared from PNHS were further fractionated by TEAE cellulose chromatography by the method of Nilsson and Muller-Eberhard (37). Two ml of

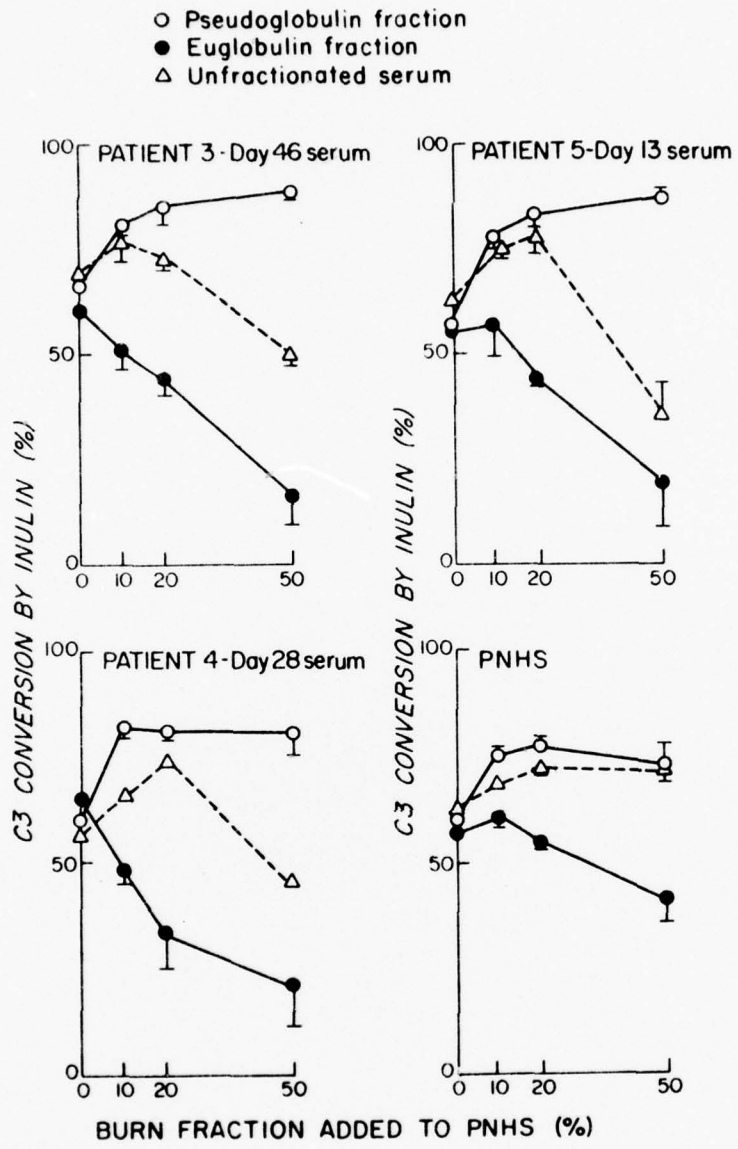


Fig. 13. C3 conversion by inulin in PNHS supplemented with unfractionated burn sera and the euglobulin or pseudoglobulin fractions of the sera. Fractions of sera from patients 3, 4, and 5 were tested; C3 conversion in the sera prior to fractionation ranged from 12% to 24% (See Fig. 12). The points represent mean values of duplicate determinations, and the vertical bars represent the standard error of the mean.

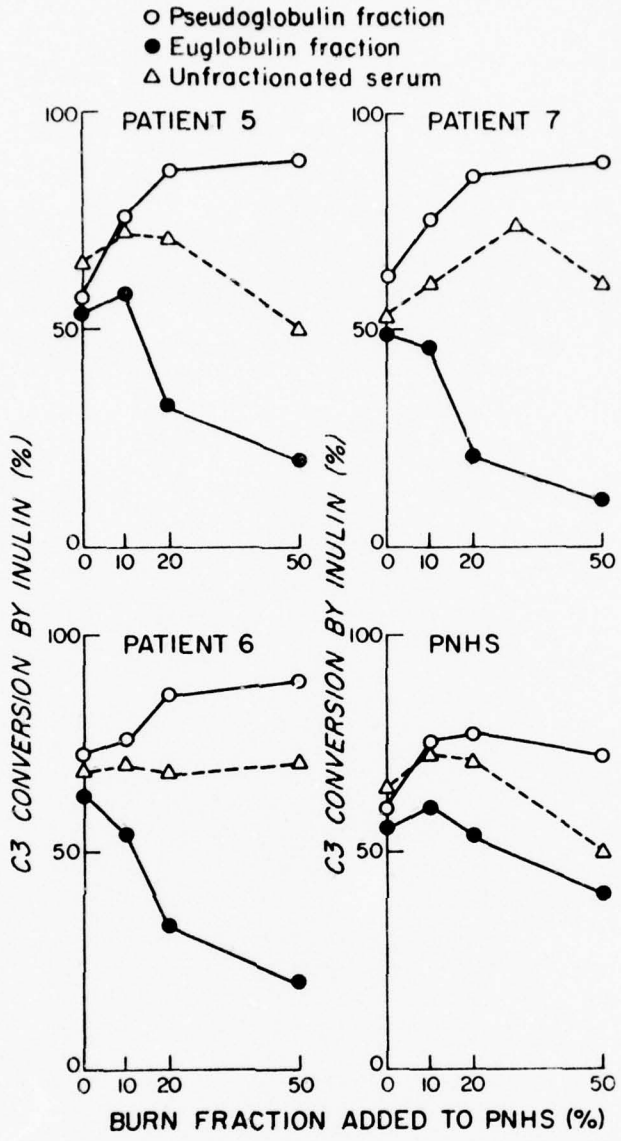


Fig. 14. C3 conversion by inulin in PNHS supplemented with unfractionated burn sera and the euglobulin or pseudoglobulin fractions of pooled sera from patients 5, 6, and 7. Sera collected on the following dates from the patients were pooled: Patient 5 - days 14, 20, and 27; Patient 6 - days 25 and 32; and Patient 7 - days 16 and 24. C3 conversion in the pooled burn sera prior to fractionation was 23% for Patient 5, 27% for Patient 6, and 23% for Patient 7. The points represent values of single determinations.

each euglobulin were dialyzed against 0.03M phosphate buffer, pH 8.1 and applied to a 2.5 x 40 cm column of TEAE equilibrated with the same buffer. A pH gradient consisting of 300 ml of 0.03M phosphate buffer, pH 8.1 and 150 ml of 0.3M NaH_2PO_4 was applied, and 5 ml fractions were collected. Four peaks were eluted, and each was concentrated to 0.5 ml and dialyzed against 0.01M phosphate buffered saline, pH 7.0. A representative graph of the protein elution is shown in Fig.15. The peaks consisted of the following fractions: (1) fractions 20 - 32, (2) fractions 50-63, (3) fractions 69 - 103, (4) fractions 104 - 120.

Equal parts of the TEAE fractions of burn or normal euglobulin and PNHS were added together, and C3 conversion by 10 mg/ml of inulin after 30 and 60 minutes of incubation at 37°C was determined. In prior experiments, C3 conversion had been measured only after 60 minutes of incubation at 37°C. The unfractionated burn euglobulin inhibited C3 conversion in PNHS to a greater extent than the normal euglobulin (Table 4). However, none of the TEAE fractions of either the normal or burn euglobulin inhibited C3 conversion in PNHS at 30 or 60 minutes. These results suggest that other chromatographic methods should be tried for fractionation of the inhibitory proteins from burn and normal euglobulin.

b. Discussion

Although the results described above do not completely exclude the possibility that reduction in C3 conversion by inulin in the burn sera is caused by a deficiency of critical normal serum proteins required for C3 conversion, they provide support for the concept that this complement abnormality is caused by an elevation of a normal regulatory protein. The protein is a euglobulin and appears to be present in greater concentration in burn sera than in normal sera.

The regulatory protein is probably not C3b inactivator (KAF) which

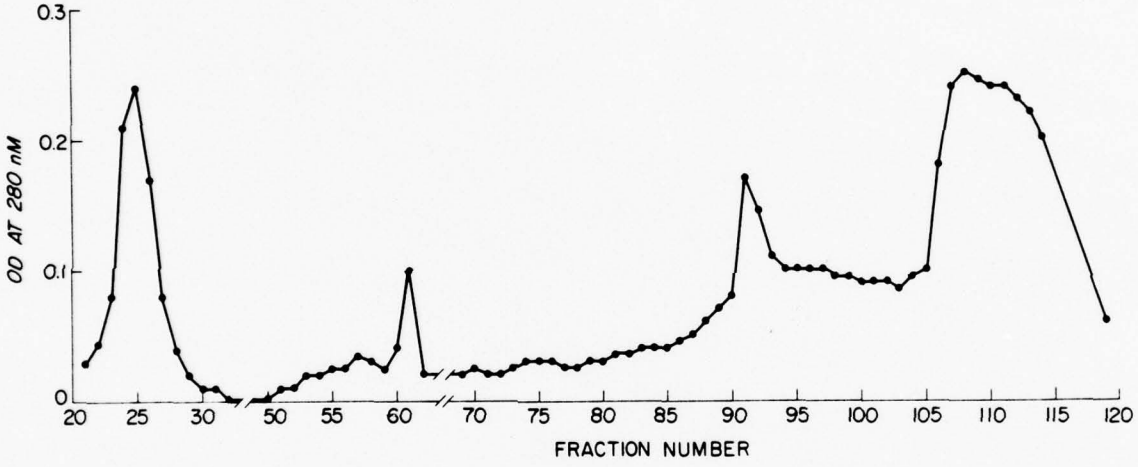


Fig. 15. TEAE chromatography of burn euglobulin. Two ml of euglobulin were applied to a 2.5 x 40 cm column. Fractions were assayed for optical density at 280nM.

Table 4. C3 Conversion by Inulin in PNHS Supplemented with TEAE Fractions of Euglobulin Prepared from Normal or Burn Sera

Reaction mixture[a]	Source of Euglobulin	Protein Concentration of Fraction mg/ml	C3 Conversion by Inulin[b] % 30 minutes	60 minutes
PNHS alone			46.4 ± 4.0	71.9 ± 3.2
Unfractionated euglobulin	Normal	16.20	7.7 ± 4.2	26.6 ± 3.4
Fraction #1		0.42	73.3 ± 1.3	91.6 ± 3.4
Fraction #2		0.80	55.0 ± 0.60	81.8 ± 1.55
Fraction #3		2.85	55.6 ± 3.3	77.0 ± 5.1
Fraction #4		0.84	50.2 ± 9.7	65.8 ± 2.25
Unfractionated euglobulin	Burn (Patient 6)	12.30	4.8 ± 2.0	4.8 ± 1.0
Fraction #1		0.38	47.3 ± 12.2	62.6 ± 3.0
Fraction #2		0.68	43.2 ± 3.85	67.0 ± 6.5
Fraction #3		4.00	44.7 ± 3.5	69.0 ± 10.3
Fraction #4		1.07	50.2 ± 1.5	72.2 ± 1.7
Unfractionated euglobulin	Burn (Patient 7)	10.0	4.1 ± 1.0	6.9 ± 2.3
Fraction #1		0.26	61.1 ± 4.3	85.8 ± 10.1
Fraction #2		0.16	62.0 ± 5.3	83.8 ± 11.1
Fraction #3		1.36	66.6 ± 10.2	74.6 ± 4.8
Fraction #4		0.18	65 ± 5.8	78.6 ± 2.9

[a] Reaction mixtures consisted of 50 ul of PNHS, 50 ul of euglobulin fraction, and 10 ul of inulin (100 mg/ml). The percent of C3 conversion at a given time period was determined by the formula a- b/a x 100 where a was the concentration of B antigen of C3 at 0 hour and b was the concentration of B antigen at 30 or 60 minutes.

[b] Each value represents the mean ± standard error of the mean of duplicate determinations.

inactivates C3b thereby inhibiting the amplification loop of the alternative pathway formed by the C3 convertases, $\overline{C3b, B}$ or $\overline{C3b, B, P}$. Quite recently a new regulatory protein, βIH, has been discovered (38, 39). βIH potentiates the inactivation of C3b by KAF and, in addition, directly inhibits C3b and the activity of the alternative pathway convertases $\overline{C3b, B}$ and $\overline{C3b, B, P}$. The protein has been isolated from normal human plasma and shown to be an asymmetric molecule with a molecular weight of 300,000 daltons. The protein is a euglobulin which is distinct from KAF. Our future studies will be directed toward determining if reduction in C3 conversion in burn sera is caused by an elevation of βIH or another as yet undescribed regulatory protein of the alternative pathway or by a deficiency of a protein or proteins required for alternative pathway activation.

B. Changes in Humoral Components of Host Defense in Patients with Non-Burn Trauma and in Septic Patients without Trauma

1. Results

In our previous published studies (8, 9), and in the studies cited in section A-1 of this report, patients with burn injury were found to have multiple alterations of complement and immunoglobulins. Some of the humoral alterations were demonstrated in the burned patients during septic episodes, whereas other alterations were clearly unassociated with systemic bacterial or fungal infection. The present investigation was undertaken to determine if the changes in complement components and immunoglobulins which were demonstrated in septic and non-septic burned patients were unique to patients with burn injury or were also observed in patients with non-burn trauma or in septic patients without trauma. It was hoped that the information obtained from the investigation would help to establish the etiology of the humoral alterations in the burned patients and to provide new information regarding the integrity of host defenses in patients with non-burn trauma.

Levels and activities of components of the classical and alternative complement pathways were first measured in the sera of ten patients with blunt or penetrating abdominal trauma. The serum samples were obtained from the patients within 6 hours following the injury. Pertinent clinical information on the patients is given in Table 5.

The classical pathway as measured functionally by total hemolytic complement (CH_{50}) was reduced in the patients' sera ($p < 0.005$) (Fig.16). Immunochemical levels of the classical components C1 and C4 were slightly decreased in the trauma sera in comparison to the normal mean levels of these components, however the reductions were not statistically significant by the Student t test at a p value of less than 0.05. The immunochemical levels of C2 and C3

Table 5. Clinical Characteristics of Patients with Abdominal Trauma

Patient no.	Type of trauma[a]	Sex[b]	Age
1	P	M	27
2	P	M	30
3	P	M	33
4	B	F	55
5	B	M	20
6	B	M	19
7	B	M	26
8	B	M	17
9	B	M	19
10	B	M	18

[a] B = blunt; P = penetrating.

[b] M = male; F = female.

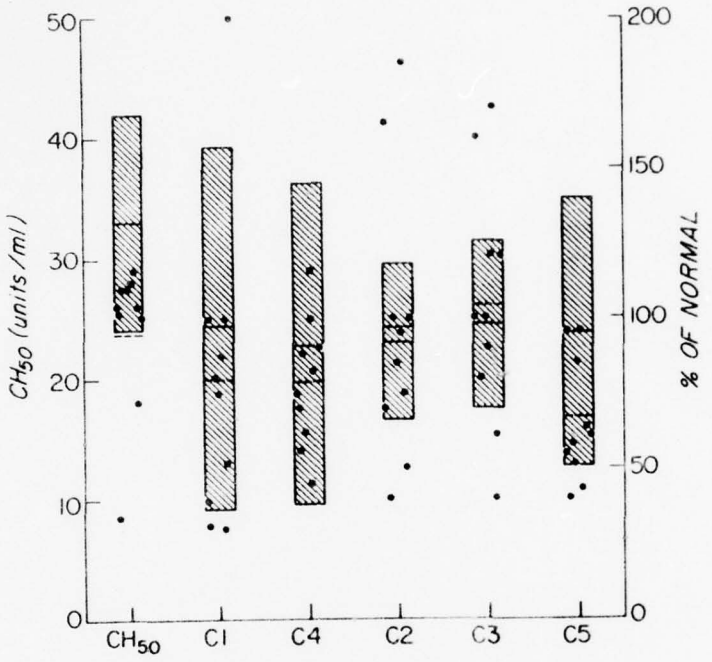


Fig. 16. Immunochemical levels and functional activity of components of the classical complement pathway in the sera of ten patients with abdominal trauma. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

were found to be normal in the sera from the trauma patients. The decrease in CH_{50} appeared to be related to the level of C5 which was found to be significantly reduced in the patients' sera ($p < 0.01$).

Immunochemical and functional activities of components of the alternative pathway were also found to be abnormal in the sera of the trauma patients. C3 conversion by CoVF and immunochemical levels of properdin and KAF were found to be significantly reduced in the sera of the trauma patients ($p < 0.025$) (Fig.17). C3 conversion by inulin and factor B levels were found to be normal in the patients' sera.

Immunoglobulin levels were also measured in the sera from the trauma patients. Levels of IgG, IgA, and IgM were reduced in the trauma sera (Fig.18). However, the only significant reduction was in the level of IgM in the patients' sera ($p < 0.01$).

Levels and activities of components of the classical and alternative complement pathways were next determined in the sera of ten septic patients without trauma. Clinical information and blood culture results on the patients are shown in Table 6. In six of the ten patients, serum samples and blood cultures were obtained on the same day. In the other four patients, serum samples were obtained one to three days after the blood cultures when the patients were still febrile. None of the patients were hypotensive at time of sample collection, however four of the patients died of septic shock on the following day.

Although three of the patients had decreased total hemolytic complement, the mean CH_{50} for the patient group was increased over the normal mean value (Fig.19). Reduction in CH_{50} in the three patients (#2, 5, 8) may have been related to their underlying diseases which were myasthenia gravis, carcinoma, and gastrointestinal bleeding. The decrease in CH_{50} was not, however, found to be related to the time of sample collection. The normal mean values for

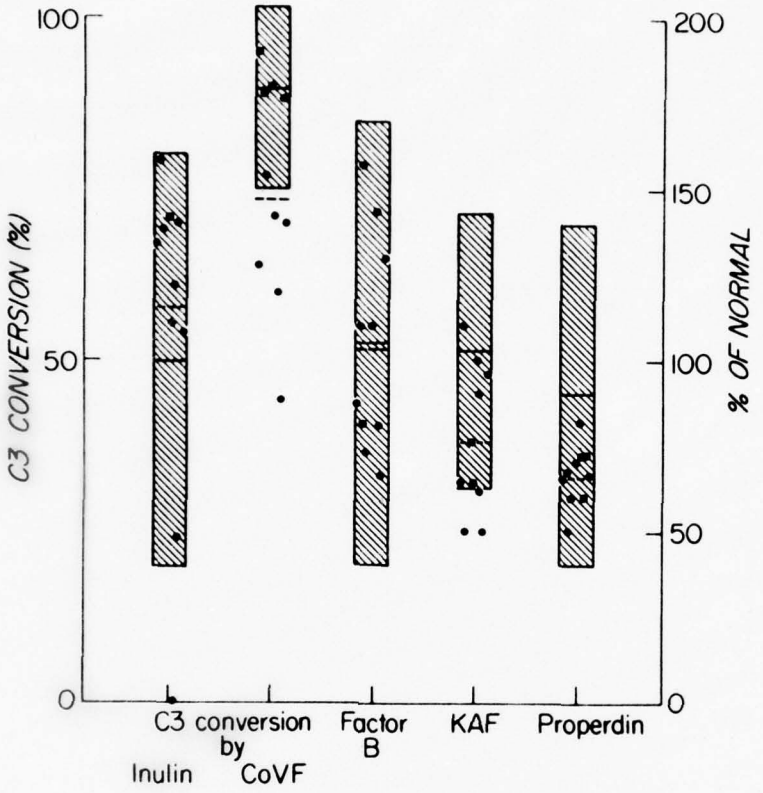


Fig. 17. Immunochemical levels and functional activity of components of the alternative complement pathway in the sera of ten patients with abdominal trauma. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

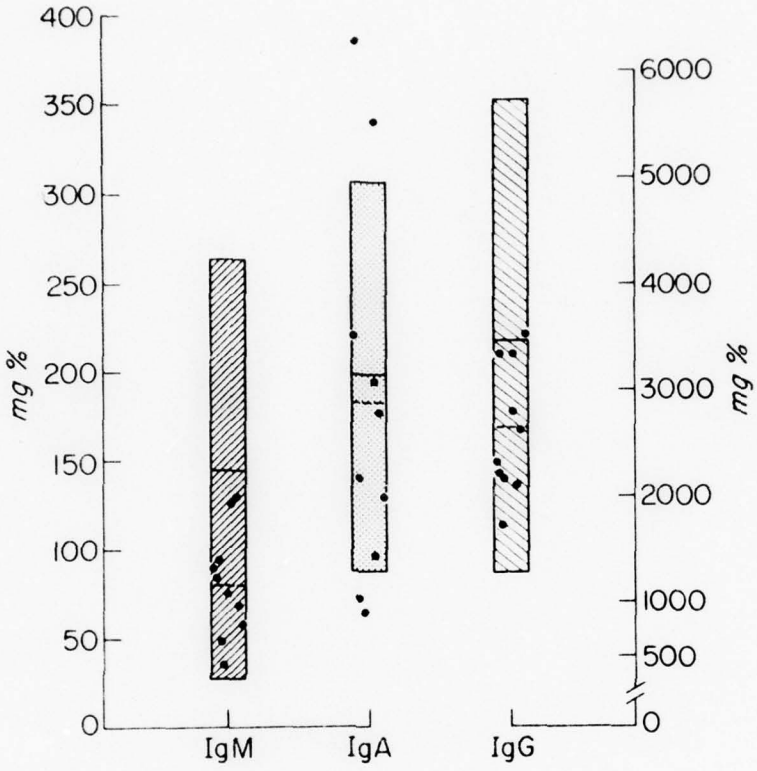


Fig. 18. Levels of IgG, IgA, and IgM in the sera of ten patients with abdominal trauma. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

Table 6. Clinical Characteristics of Septic Medical Patients without Trauma

Patient no.	Sex[a]	Age	Infecting Microorganism	Underlying Disease	Days[b] Post infection
1	M	74	Serratia marcescens	chronic obstructive lung disease	0
2	F	54	Klebsiella pneumoniae	myasthenia gravis	3
3	F	52	Serratia marcescens	organic brain syndrome with ruptured appendix	3
4	M	73	Enterobacter cloacae	intercerebral hemorrhage	0
5	M	62	Escherichia coli	carcinoma of lung	2
6	F	38	Staphylococcus aureus	none	1
7	M	54	Streptococcus pneumoniae	pneumonia	0
8	M	30	Escherichia coli	chronic alcoholism with hepatitis, gastro-intestinal bleeding with peritonitis	0
9	M	60	Staphylococcus aureus	chronic alcoholism with hepatitis	0
10	F	39	Streptococcus pneumoniae	pneumonia	0

[a] M = male; F = female.

[b] Days post infection indicates the number of days after the blood culture was obtained that the serum sample was collected. Zero indicates that the culture and serum sample were obtained on the same day.

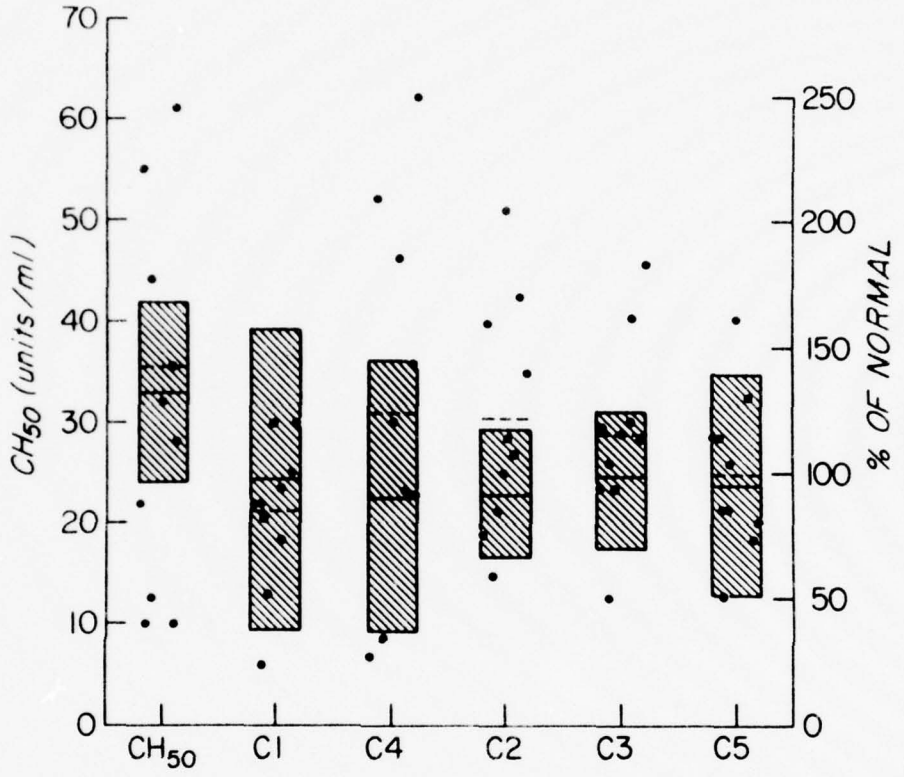


Fig. 19. Immunochemical levels and functional activity of components of the classical complement pathway in the sera of ten septic non-trauma patients. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

immunochemical levels of C1, C4, C2, C3, and C5 in the patient group were equivalent to the normal mean values for these components.

Conversion of C3 by inulin or CoVF was normal in the septic non-trauma patients (Fig. 20). Immunochemical levels of factor B, KAF, and properdin were normal or elevated in the patients' sera. These results indicated that the alternative complement pathway was also normal in the sera of this patient population.

Immunoglobulin levels were also measured in the patients' sera (Fig.21). The level of IgG in the sera was normal, whereas the level of IgA was markedly increased. The level of IgM was significantly decreased in the sera of the septic patients when compared to normal values (p value < 0.025).

2. Discussion

Multiple abnormalities of humoral components of host defense have been characteristically observed following burn injury. We previously determined the temporal sequence of alterations of complement, immunoglobulins, and opsonic factors in thermally injured patients (7-9). In addition, we have identified those humoral abnormalities which were related to the severity of the burn injuries and those associated with septicemia. We hypothesized that the changes in humoral factors which were unrelated to burn size might not necessarily be a direct consequence of burn injury but might be the result of trauma in general. Moreover, it was of importance to determine if complement consumption which was found to be associated with septicemia in the burned patients was merely the consequence of the infection per se or resulted from a synergistic effect between the infection and the burn injury.

Abnormalities of both the classical and alternative complement pathways were found to occur immediately following severe blunt or penetrating abdominal trauma. Conversion of C3 by CoVF which is a functional measurement of the alternative pathway was reduced in the sera of the trauma patients, and

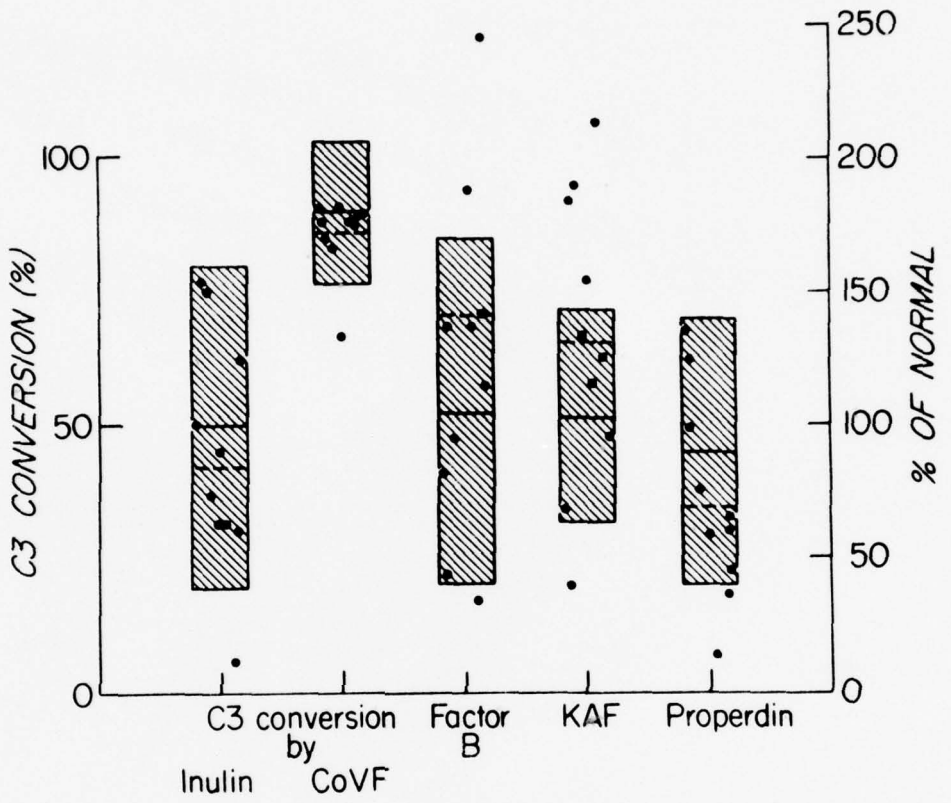


Fig. 20. Immunochemical levels and functional activity of components of the alternative complement pathway in the sera of ten septic non-trauma patients. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

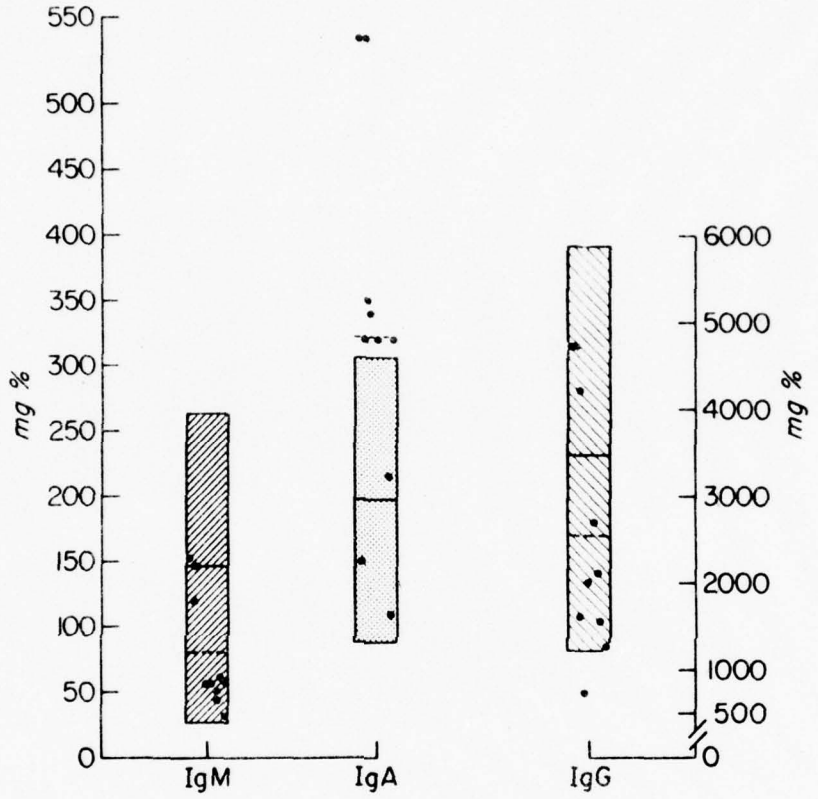


Fig. 21. Levels of IgG, IgA, and IgM in the sera of ten septic non-trauma patients. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 SD) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

decrease in the level of properdin and KAF was also demonstrated in the trauma sera. CH_{50}, a functional measurement of the classical pathway, and the immunochemical level of C5 were also decreased. Conversion of C3 by inulin and levels of factor B, C1, C4, C2, and C3 were found to be normal in the patients' sera.

It is unclear why C3 conversion by CoVF was reduced in the trauma sera, whereas C3 conversion by inulin in the sera was normal. C3 conversion by inulin requires factors B, $\overline{D}$, initiating factor, C3, and magnesium ions (17, 18), and C3 conversion by CoVF requires factors B, $\overline{D}$, magnesium ions, and an additional euglobulin termed factor E(40). It is possible that the sera from the trauma patients was deficient in factor E in addition to properdin and KAF. Further studies are required to determine the identity and significance of deficient or abnormally functioning alternative pathway components in the sera from trauma patients.

Reduction in C5 in the trauma sera could have resulted from decreased KAF. KAF inactivates C3b which is required for formation of the alternative pathway C5 convertase, $\overline{C3b, B, P}$ (18, 24). A decrease in KAF would cause an increase in C3b which through formation of the C5 convertase would result in increased turnover of C5. Activation of C5 leads to consumption of C6 to C9, and therefore these components are probably also reduced in the trauma sera.

A decrease in serum IgM was also observed in the trauma patients. A concommitant reduction in serum IgM (41) and properdin (42) levels has previously been demonstrated in splenectomized children. Decrease in IgM level in patients with sickle cell disease has correlated well with splenic function as measured by spleen scanning (43). In addition, children who undergo splenectomy for various hemolytic diseases or for traumatic rupture of the spleen have decreased IgM levels, supporting the concept that there

is a correlation between splenic mass and concentration of this immunoglobulin (44). The reduction in IgM concentration does not appear to be the result of decreased splenic IgM synthesis, since IgM synthesis could be adequately assumed by other reticuloendothelial and lymphoid tissue. All of our patients had splenic trauma and all of those that survived subsequently underwent splenectomy. Thus, the decrease in IgM concentration in the sera of our patients may have been related to spleen damage. Our future studies will be designed to determine the correlation between spleen function and IgM level in the trauma patients in an attempt to determine the mechanism of the reduction in the level of this immunoglobulin. In addition, it will be of interest to determine the relationship between splenic function and reduction in properdin, KAF, and C5 in the trauma patients.

There have been virtually no investigations into the sequential effect of trauma on humoral host defenses. In addition, there are few studies that provide experimental evidence that trauma does indeed increase susceptibility to infection. Conolly et al. found significantly more infections in rabbits injected subcutaneously with P. aeruginosa and subjected to mechanical trauma to the thigh than control animals that did not receive this injury (45). Cuthbertson et al. (46) subjected patients with bone fractures to varying degrees of cold temperature, 20°C and 30°C respectively. Levels of IgG were the same in both groups, however some minor fluctuations in IgM level were observed during ten days in the group subjected to the lower temperature. The changes were not impressive, since all variations were within the normal range.

Studies on the effects of wound trauma on humoral antibody responses are also limited. Havens et al. (47) could detect no reduction in the antibody response to diphtheria toxoid in shick-negative wounded patients. Balch showed that the secondary response to tetanus toxoid was normal in trauma patients (48).

Trauma has been shown to depress clearance by the reticuloendothelial system (49-54), phagocytosis by neutrophils (55, 56), and cell mediated immunity (57). These changes are of short duration lasting during the first few days after trauma and are followed by restoration of normal activity. The cause and significance of these cellular abnormalities is unknown.

It is obvious from our pilot study demonstrating changes in humoral components of host defense in trauma patients that further comprehensive studies on larger numbers of patients are needed. It will be important to determine the cause and significance of the humoral abnormalities which occur following trauma and to determine the duration of the abnormalities.

Our study did not identify any reduction in components of the classical or alternative complement pathways in septic patients without trauma, suggesting that complement consumption in the septic burned patients was a result of synergism between the infection and the trauma. McCabe (58) previously showed that C3 levels were decreased in medical patients with septic shock, as compared to C3 levels in patients with uncomplicated bacteremia. The frequency of occurrence of shock or fatal outcome paralleled the degree of lowering of C3 levels in the patients. Fearon et al. (30) subsequently demonstrated decreased immunochemical levels of factor B, properdin and C3, C5, C6, and C9 in patients with septic shock in comparison to those patients with uncomplicated bacteremia, suggesting that consumption of complement occurred via the alternative pathway. Mean levels of classical components C1, C4, and C2 in the bacteremic patients in whom shock subsequently developed did not differ from those in patients with uncomplicated bacteremia.

Although none of our medical septic patients was hypotensive at the time of serum sample collection, 4 of the 10 patients died of septic shock on the following day, and no complement consumption was demonstrated in these patients. In addition, consumption of the classical complement pathway was

demonstrated in septic burned patients who were not hypotensive and survived. These observations suggest that classical complement pathway consumption associated with septicemia is unique to the thermally injured patient and is probably related to the increased number of microorganisms circulating in the blood of the burned patients.

C. Normal Human Serum Opsonins for Opportunist Microorganisms

1. Studies to determine the mechanisms of complement activation and the role of immunoglobulin in opsonization of E. coli 075 and other aerobic opportunist microorganisms

a. Results

In our previous studies, the classical complement pathway was shown to be utilized exclusively in burned patients during opsonization of E. coli 075. Serum opsonic activity for E. coli 075 and classical pathway activity were decreased concurrently in the burned patients during the first week postburn, despite normal alternative pathway activity as measured by C3 conversion by inulin. This observation led us to initiate studies to answer two important questions related to the definition of serum proteins required for opsonization of E. coli 075 and to alterations of these humoral factors in burned patients. The questions are as follows: (1) Was the classical pathway utilized in the burn sera during opsonization of E. coli 075 because the alternative pathway could not be activated, or was the classical pathway utilized preferentially?; (2) Why was the switch to an apparently functional alternative pathway and thus normal opsonization not possible, when classical pathway activity was decreased in the burn sera?

Our initial experimental studies to answer these questions were directed toward determining if E. coli 075 was, in fact, capable of activating the alternative complement pathway. The lipopolysaccharide (LPS) portion of the cell wall of gram-negative bacilli, such as E. coli 075, is known to be responsible for its anticomplementary activity (59-61). The moiety of the LPS which is responsible for complement activation is controversial. There is some experimental evidence to indicate that it is the lipid A moiety of the LPS which is responsible for this biological activity (62, 63). Because E. coli 075

by the Student t test.

might be unique in the mechanism of complement activation it was capable of initiating, we also investigated the ability of other representative gram-negative bacilli and a gram-positive opportunist, S. aureus, to activate the alternative complement pathway. In addition, we included S and R forms (Rb and Re) of S. minnesota containing varying amounts of the polysaccharide portion of the LPS in an attempt to determine the moiety of the cell wall which was responsible for alternative pathway activation. S. minnesota S form contains a complete LPS, the Rb mutant lacks the O antigen and acetylglucosamine attached to the terminal glucose but contains the rest of the basal core, and the Re mutant contains only KDO and lipid A.

Washed heat-killed cells of E. coli 075, P. mirabilis 7056, P. aeruginosa 73044, and S. minnesota chemotypes (S, Rb, and Re), and S. aureus 502A were tested for their ability to activate the alternative complement pathway. Intact bacterial cells were used rather than purified LPS and lipid A in these initial experiments, since there is evidence that the method of purification of the cell wall components may influence their biological activities (63). Parameters of alternative pathway activation were (a) conversion of C3 in 10 mM ethylene glycol tetraacetic acid (EGTA)-treated human serum supplemented with 2.5 mM $MgCl_2$, (b) lysis of glutathione-treated human erythrocytes (GSHE) in the presence of human serum, and (c) C3 to C9 consumption in C4 deficient guinea pig serum.

To determine the minimal concentration of magnesium ion (Mg^{2+}) required to allow alternative pathway activation in 10 mM EGTA-treated normal human serum (NHS), the EGTA-treated serum was supplemented with increasing concentrations of $MgCl_2$, and C3 conversion by inulin and BSA-anti-BSA immune precipitates was measured after 60 minutes of incubation at 37°C. Addition of 2.5 mM $MgCl_2$ to the EGTA-treated serum resulted in C3 conversion by inulin equal to that in untreated NHS. Immune precipitates did not initiate C3 conversion in this serum

(Fig.22). EGTA-treated NHS supplemented with 2.5 mM $MgCl_2$ also did not promote lysis of sensitized erythrocytes (EA) providing further evidence that the classical complement pathway was effectively blocked. Addition of 5 or 10 mM $MgCl_2$ to the EGTA-treated serum resulted in C3 conversion by both inulin and immune precipitates and 5 to 20% lysis of EA. Therefore, EGTA-treated NHS supplemented with 2.5 mM $MgCl_2$, hereafter referred to as EGTA-NHS-Mg^{2+}, was used in subsequent experiments.

Washed cells of P. aeruginosa, P. mirabilis, S. minnesota S form, E. coli, and S. aureus converted C3 in untreated NHS, although none of the bacterial strains was as active as inulin (Fig.23). In contrast, S. minnesota chemotypes Rb and Re were less efficient than the other bacterial strains in converting C3 in NHS. With the exception of S. aureus and S. minnesota Re, no statistical difference was observed between conversion of C3 by the other bacterial strains or inulin in EGTA-NHS-Mg^{2+} or untreated NHS. S. minnesota Re did not convert C3 to any extent in EGTA-NHS-Mg^{2+}, and C3 conversion by S. aureus in this serum was significantly reduced at the 60 and 90 minutes time intervals when compared to C3 conversion by this bacterial strain in untreated NHS.

Although none of the bacterial strains was as efficient as inulin in initiating lysis of GSH E, E. coli and P. mirabilis were more active than the other bacterial strains in this test system (Fig.24). S. minnesota S and Rb, P. aeruginosa, and S. aureus were intermediate in initiation of lysis of GSH E, and S. minnesota Re was inactive in the test system.

With the exception of S. minnesota Re, all of the other bacterial strains initiated C3 to C9 consumption in C4 deficient guinea pig serum (Table 7). E. coli, P. mirabilis, and S. minnesota S were the most efficient activating substances followed by P. aeruginosa, S. aureus, and S. minnesota Rb.

These results indicated that E. coli 075 and the other gram-negative aerobic

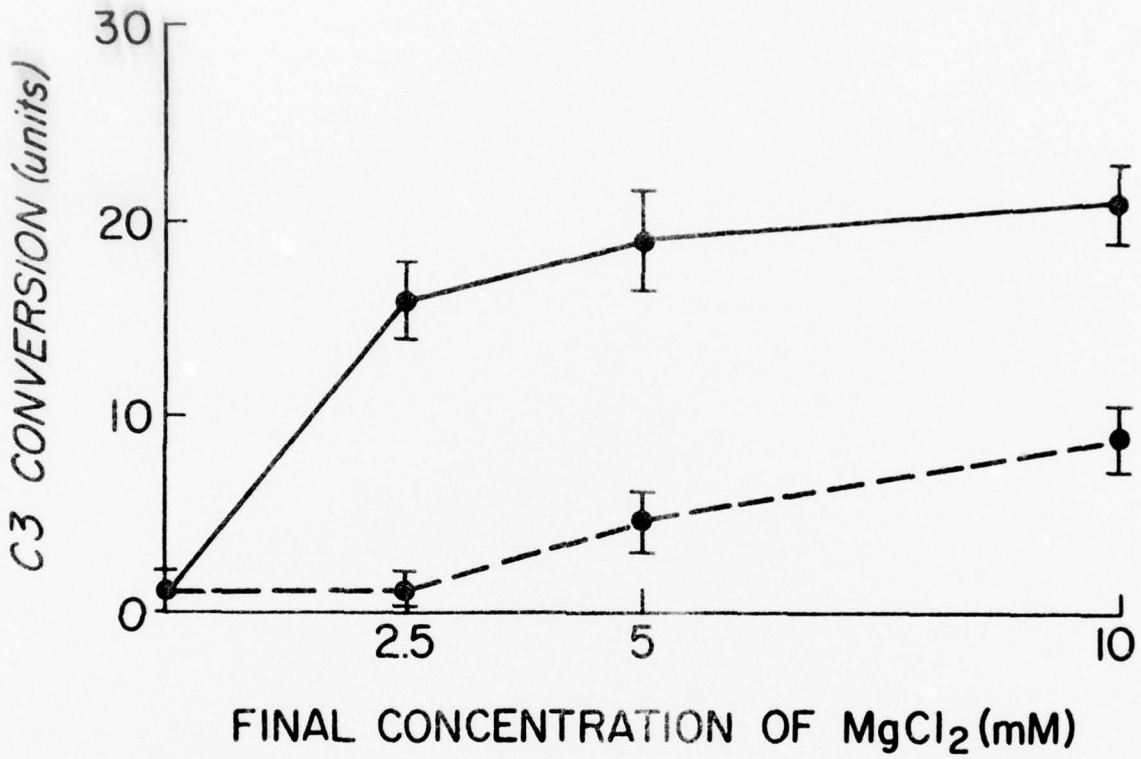


Fig. 22. C3 conversion by inulin (10 mg/ml) or immune precipitates (25 mg/ml) in 10 mM EGTA-treated NHS supplemented with increasing concentrations of $MgCl_2$. The solid line represents C3 conversion by inulin, and the dotted line represents C3 conversion by immune precipitates. The vertical bars represent the standard error of the mean of the triplicate determinations. C3 conversion in untreated NHS by inulin was 19 $\pm$ 3 units and by immune precipitates was 20 $\pm$ 2 units.

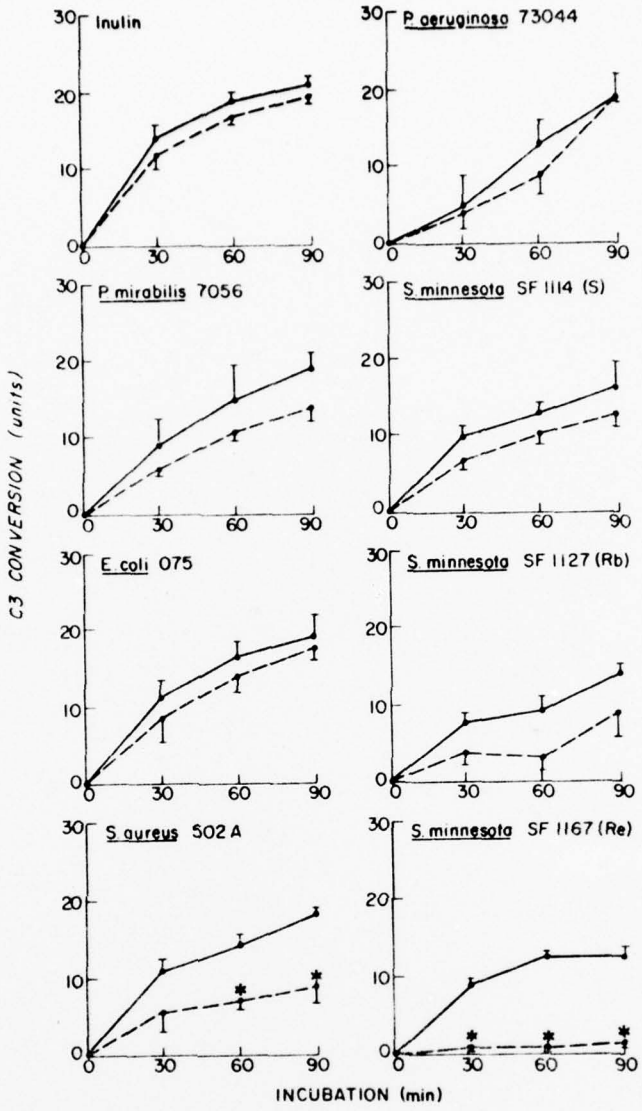


Fig. 23. C3 conversion by inulin (10 mg/ml) or washed bacterial cells (1.0×10^9 cells/ml) during 90 minutes of incubation. The solid line represents C3 conversion in NHS, and the dotted line represents C3 conversion in NHS-EGTA-Mg^{2+}. The vertical bars represent the standard error of the mean of triplicate determinations. *$p < 0.01$ vs. untreated NHS at the same time period. Statistical analysis was performed by the Student t test.

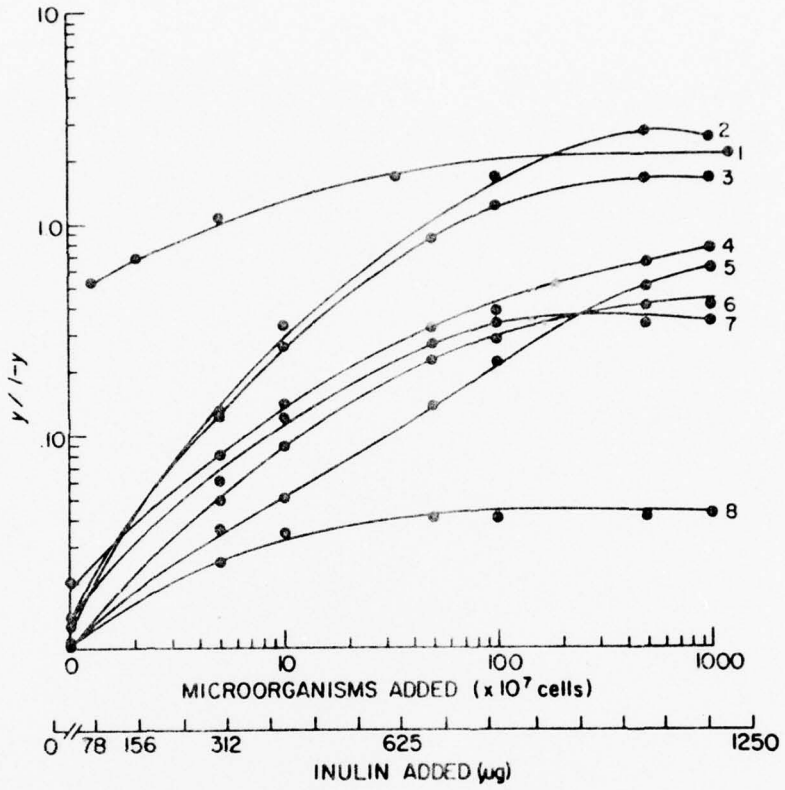


Fig. 24. Lysis of GSH E initiated by inulin (10 mg/ml) or washed bacterial cells (1.0×10^9 cells/ml). Y/1-Y of 1.0 corresponds to 50 percent lysis. Lysis in the absence of an activating substance was 0.041. Points represent the average of duplicate determinations. Activating substances were as follows: (1) Inulin, (2) E. coli 075, (3) P. mirabilis 7056, (4) S. minnesota SF 1114 (S), (5) S. minnesota SF 1127 (Rb), (6) P. aeruginosa 73044, (7) S. aureus 502A, and (8) S. minnesota SF 1167 (Re).

Table 7. Consumption of C3 to C9 Hemolytic Activity in C4 Deficient Guinea Pig Serum by Inulin (10 mg/ml) or Washed Bacterial Cells (1.0×10^9 cells/ml)

Activating substance	C3-C9 Consumption[a] %
Inulin	92.05 ± 1.15
E. coli 075	82.85 ± 1.95
P. mirabilis 7056	76.60 ± 0.60
S. minnesota SF 1114 (S)	67.90 ± 0.10
P. aeruginosa 73044	55.15 ± 2.85
S. minnesota SF 1127 (Rb)	50.0 ± 4.30
S. aureus 502A	38.50 ± 9.6
S. minnesota SF 1167 (Re)	9.50 ± 1.20
Saline	4.50 ± 2.0

[a]Mean values ± the standard error of the mean of triplicate determinations are represented.

bacilli which were tested were capable of activating the alternative complement pathway in normal serum. In contrast, S. aureus was less efficient in initiating alternative pathway activation, although it was very active in initiating the classical pathway. Preliminary results utilizing cell wall mutants of S. minnesota indicated that the polysaccharide portion of the cell wall of gram-negative bacilli was responsible for alternative pathway activation, whereas the lipid A moiety was responsible for activation of the classical pathway. Thus, gram-negative bacilli with an outer and thus exposed polysaccharide cell wall moiety, such as E. coli 075, should be capable of activating the alternative pathway in either normal or burn sera. A large amount of immune IgG antibodies might, however, divert complement activation from the alternative to the classical pathway by virtue of the ability of the antibodies to bind to the Clq portion of Cl. We hypothesized that these antibodies might exist naturally in human sera or be produced as a result of the administration of P. aeruginosa vaccine, since all of our burned patients had received the vaccine. The antibodies in addition to diverting activation to the classical pathway might block activation of the alternative pathway, thereby explaining why opsonization of E. coli 075 did not proceed normally in the burn sera when classical pathway activity was decreased. Immune IgG antibacterial antibodies which trigger classical pathway activation are heat-stable and promote phagocytosis and intracellular killing of bacteria in the absence of complement (26, 27, 64). We therefore measured the heat-stable opsonizing activity for E. coli 075 of pooled normal human serum (PNHS), individual normal sera, and sera from burned patients who had received a complete course of vaccine therapy.

The sera were heated at 56°C for 30 minutes, and opsonic activity for E. coli 075 at a 2% serum concentration was measured. This concentration was utilized because it was found to be the minimal amount of PNHS required to

promote maximal intracellular killing. PNHS had no residual opsonic activity for E. coli 075 after heat treatment when compared to unheated PNHS (Fig. 25). Increasing the concentration of heated PNHS added to the opsonic assays did not substantially improve its opsonic activity (Fig.26). The opsonic activity of sera from four individual normal donors was also found to be eliminated by heat treatment (Fig.27). The opsonic activity of serial serum samples from four burned patients who had received 25 ug/kg of P. aeruginosa vaccine at admission, 4 and 8 days thereafter, and then at weekly intervals was tested before and after heating. Prior to heat treatment, opsonic activity of the sera was equivalent to the opsonic activity of the normal sera, and no residual opsonic activity remained after heating. These results indicated that heat-stable opsonizing antibodies for E. coli 075 were not present in large amounts in the sera from either the normal controls or the vaccinated patients. Utilization of the classical pathway in the burn sera during opsonization of E. coli 075 could therefore not be explained by the presence of immune antibacterial antibodies and, in fact, we questioned if antibodies played any role in opsonization of this microorganism.

In an attempt to provide preliminary information regarding the requirement for immunoglobulin in opsonization of E. coli 075, we initiated experiments to compare the opsonic activity of PNHS and hypogammaglobulinemic sera. The hypogammaglobulinemic sera were obtained from three different donors and contained minimal amounts of IgG, IgA and IgM as outlined in the legend to Fig. 28. The opsonic activity of the hypogammaglobulinemic sera was equivalent to the opsonic activity of PNHS (Fig. 28). These results suggested a minimal role, if any, for immunoglobulin in opsonization of E. coli 075.

The opsonic activity of highly purified normal human IgG for E. coli 075 was also measured. Normal IgG was purified by the method of Sober et al. (65) and dialyzed against 0.01 M phosphate-buffered saline, pH 7.0. By immunoelectrophoretic analysis using anti-whole human serum, the preparation contained a

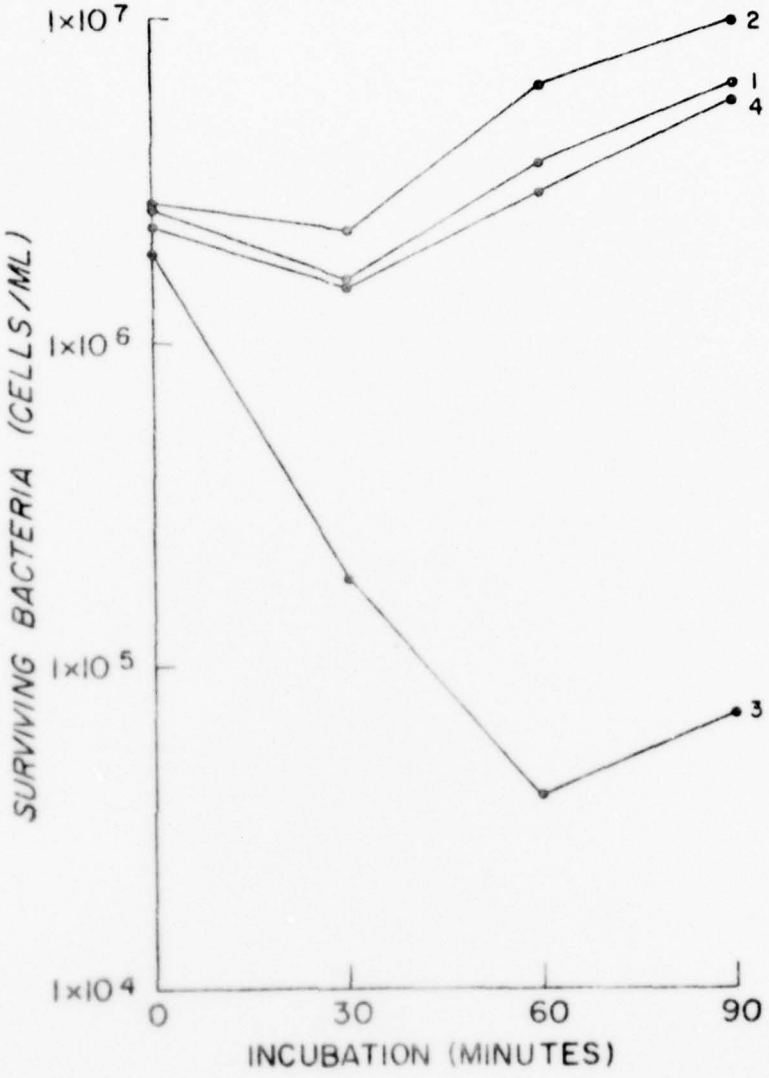


Fig. 25. Opsonic activity of heated pooled normal human serum (PNHS) for E. coli 075. The following reaction mixtures were tested: (1) PNHS and bacteria, (2) leukocytes and bacteria, (3) PNHS, leukocytes and bacteria, (3) PNHS, leukocytes, and bacteria, and (4) PNHS (56°C, 30 minutes), leukocytes, and bacteria. The serum concentration used in the reaction mixtures was 2%.

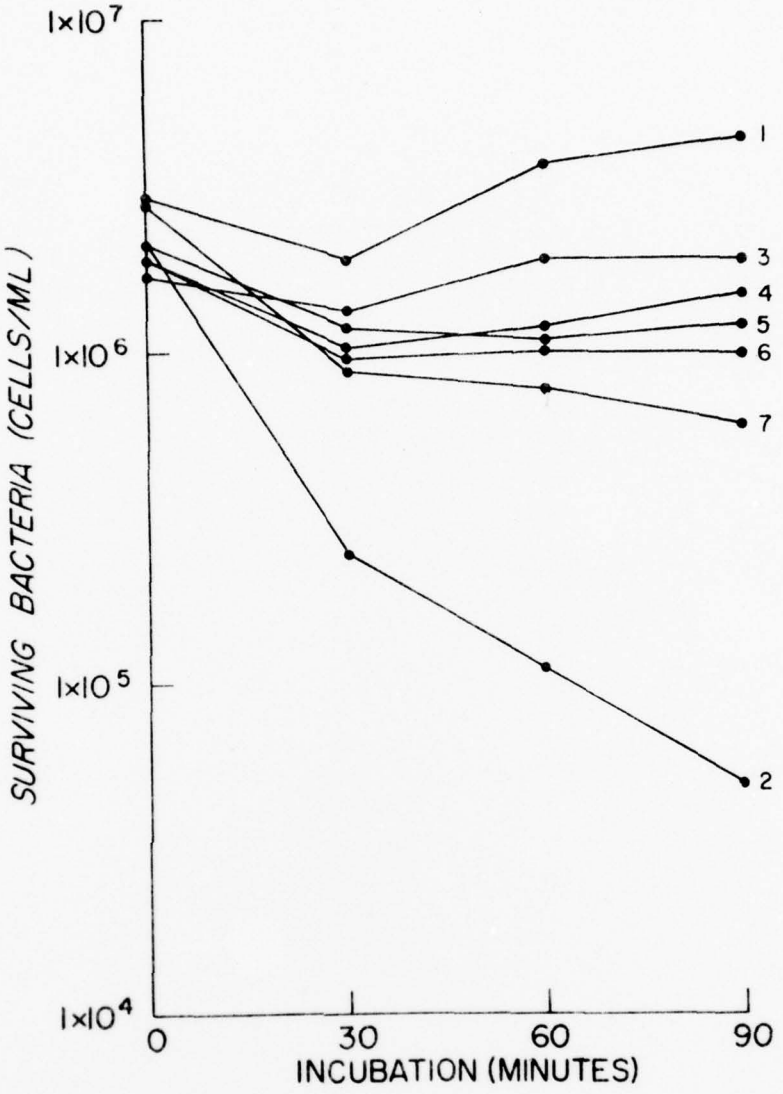


Fig. 26. Opsonic activity of increasing concentrations of heated pooled normal human serum (PNHS) for E. coli 075. The following reaction mixtures were tested: (1) leukocytes and bacteria, (2) unheated PNHS (2%), leukocytes, and bacteria, (3) 2% △ PNHS (56°C, 30 minutes), leukocytes, and bacteria, (4) 5% ⎯ PNHS, leukocytes and bacteria, (5) 10% ⟍ PNHS, leukocytes, and bacteria, (6) 20% △ PNHS, leukocytes, and bacteria, and (7) 30%/⟍PNHS, leukocytes, and bacteria.

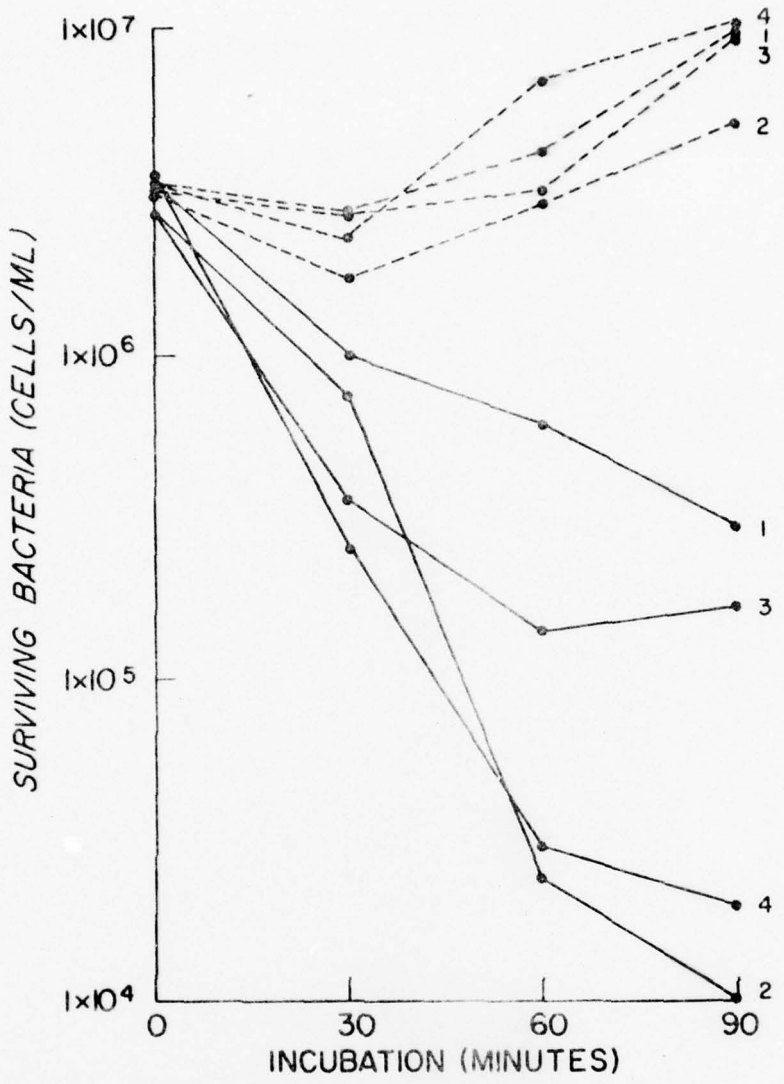


Fig. 27. Opsonic activity of heated and unheated individual normal sera for E. coli 075. The solid lines represent unheated sera and the dotted lines represent heated sera. The numbers following the lines represent each normal individual's number. Reaction mixtures consisted of serum, leukocytes and bacteria. The serum concentration used in all of the reaction mixtures was 2%.

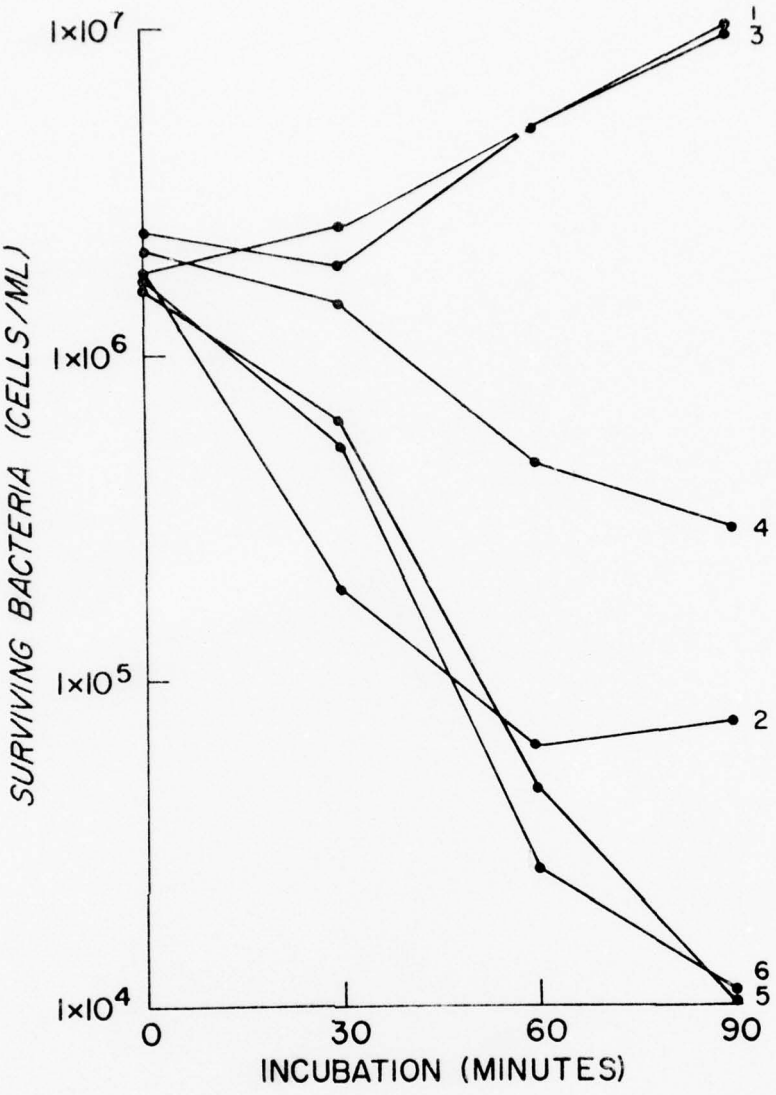


Fig. 28. Opsonic activity of hypogammaglobulinemic sera for E. coli 075. The following reaction mixtures were tested: (1) PNHS and bacteria, (2) PNHS, bacteria, and leukocytes, (3) leukocytes and bacteria, (4) hypogammaglobulinemic serum (HS_1), leukocytes, and bacteria, (5) HS_2, leukocytes, and bacteria, and (6) HS_3, leukocytes, and bacteria. HS_1 contained 780 mg% of IgG, < 15 mg% of IgM, and < 24 mg% of IgA. HS_2 contained 300 mg% of IgG, 16 mg% of IgM, and < 24 mg% of IgA. HS_3 contained 240 mg% of IgG, 29 mg% of IgM, and 24 mg% of IgA. The serum concentration used in the reaction mixtures was 2%.

single arc migrating in the gamma region. By radial immunodiffusion, the preparation was found to contain 10.25 g/100 ml of IgG; IgA and IgM were undetectable in the preparation. The preparation did not promote phagocytosis of E. coli 075 by leukocytes at a 10% concentration (10.25 mg/assay) (Fig.29). These results provided further support for the concept that IgG antibodies capable of promoting opsonization of E. coli 075 in the absence of complement were not present in normal human serum.

In addition to the studies described above, we have continued our efforts to obtain recognized components of the alternative complement pathway in purified form from normal human serum. We have successfully purified human C3, properdin, and factor B, and our efforts during this year have focused upon obtaining factor $\bar{D}$ in purified form. Now that we have shown that E. coli 075 and P. aeruginosa 73044 activate the alternative pathway during opsonization and probably only activate the classical pathway under selected experimental conditions, we plan to continue with our approach to define the alternative pathway proteins required for opsonization by adding multiple combinations of purified proteins together and determining the extent of phagocytosis and killing of the microorganisms by leukocytes.

Two different methods have been used to purify human factor $\bar{D}$. The first method involved separation of pseudoglobulin, fractionation on BioRex 70, fractionation on CM cellulose, and gel filtration on Sephadex G-150 (40). A second method involved fractionation of pseudoglobulin on BioRex 70, fractionation on CM cellulose, and gel filtration on Sephadex G-75 (22). Active fractions were identified by their ability to convert C3 in the presence of purified factor B (66). This assay system was found to be more sensitive than the other method which we had been using, i.e., restoration of the C3 converting activity of factor $\bar{D}$ depleted serum. The purification methods described above were found to be inadequate as low yields of the protein were obtained, and the preparations were contaminated with 20 or more different proteins. Purity of the preparations was determined by polyacrylamide disc gel electrophoresis.

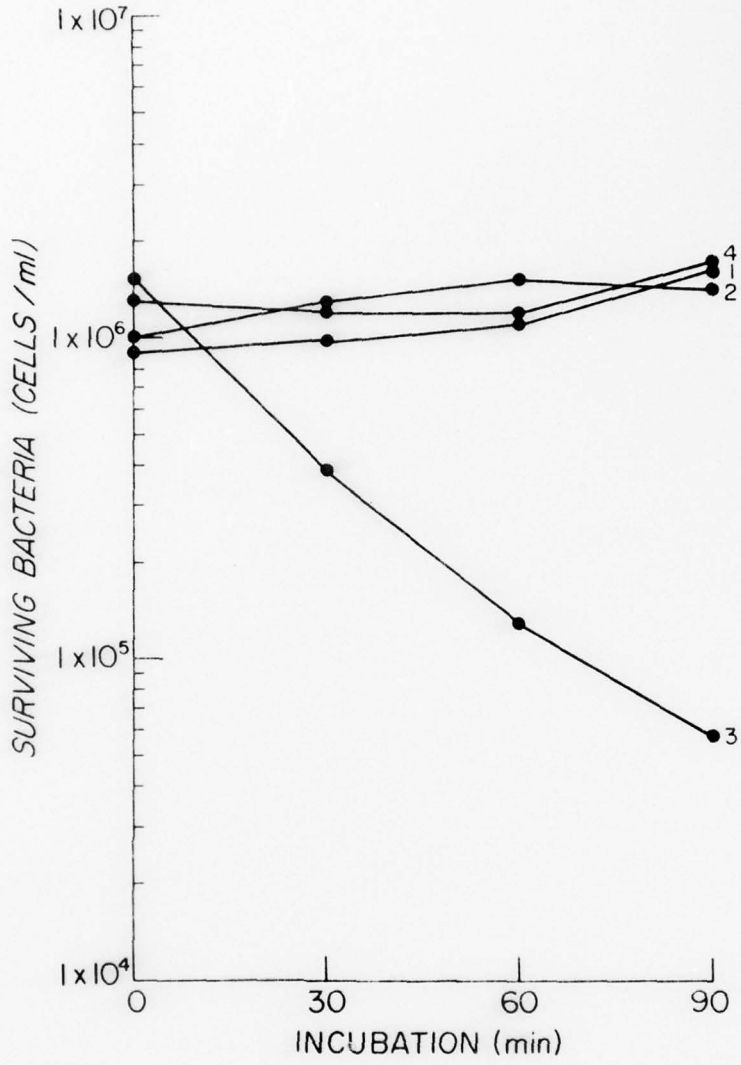


Fig. 29. Opsonic activity of purified normal human gamma globulin for E. coli 075. The following reaction mixtures were tested: (1) PNHS and bacteria, (2) leukocytes and bacteria, (3) PNHS, bacteria, and leukocytes, and (4) normal gamma globulin, bacteria, and leukocytes. The normal gamma globulin was added to the reaction mixture at a concentration of 10% and contained 10.25 g/100 ml of IgG and no detectable IgA or IgM.

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A third method of purification of factor $\bar{D}$ was found to be more effective than either of the first two methods. EDTA-treated human serum was passed over a Sephadex G-75 column equilibrated in 0.15 M NaCl, 0.002M EDTA, pH 7.3. Factor $\bar{D}$, which has a molecular weight of 24,000, was eluted at the beginning of the second protein peak (Fig.30). The preparation was found to be functionally active and, at a protein concentration of 50 ug, contained 7 bands on alkaline polyacrylamide electrophoresis. Our attempts to cut the acrylamide gels and elute the proteins to determine which band corresponds to active factor $\bar{D}$ have been unsuccessful. I have written and received information from Dr. Douglas Fearon at Harvard Medical School and Dr. Peter Lachmann at MRC in Cambridge regarding their methods for purification of factor $\bar{D}$. They have informed me that they also have had great difficulty in purifying this protein and provided helpful suggestions. The reasons for the difficulty in purifying factor $\bar{D}$ are related to (a) its low concentration in normal human serum, (b) its affinity for chromatographic resins making it difficult to elute in high yield, and (c) the unavailability of antiserum to factor $\bar{D}$ making assays for its detection time consuming and tedious.

b. Discussion

The data obtained from our investigation suggest that the classical pathway was utilized in the burn sera during opsonization of E. coli 075 because alternative pathway proteins required for opsonization of this microorganism were not functioning properly. This conclusion is based on several pieces of experimental evidence as follows: (a) Intact cells of E. coli 075 were found to be capable of activating the alternative complement pathway. (b) Preliminary experiments using S. minnesota cell wall mutants indicated that the polysaccharide portion of the lipopolysaccharide is responsible for

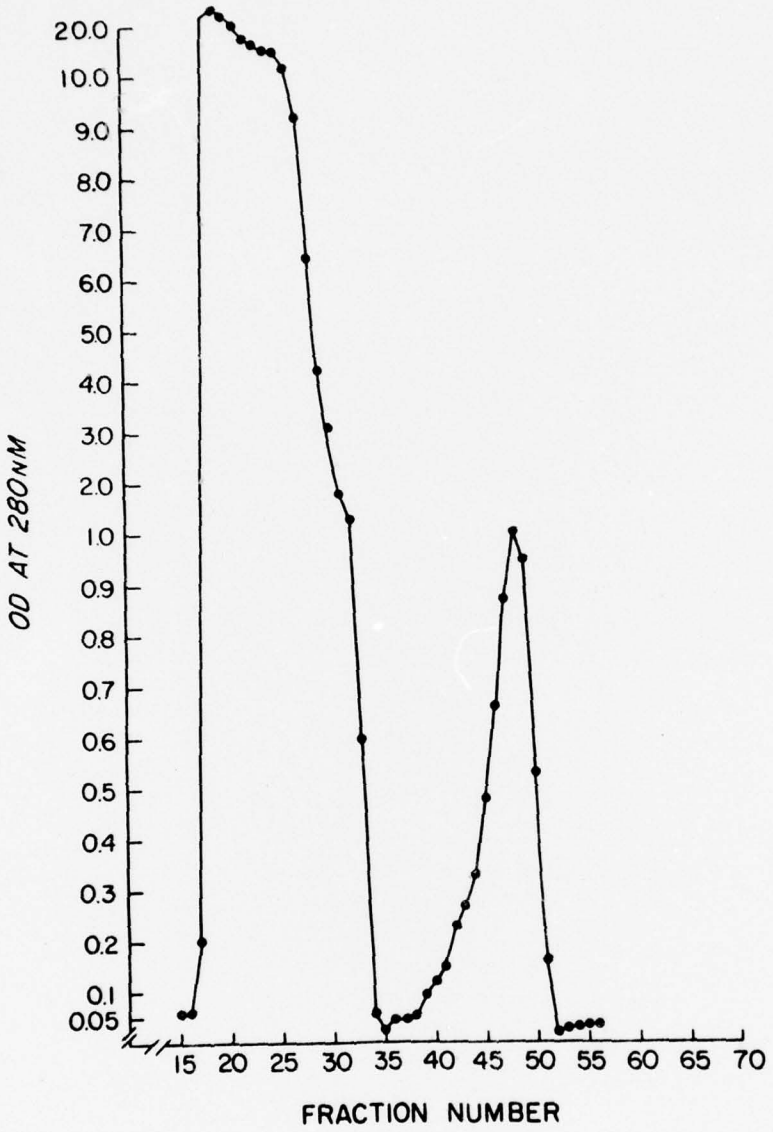

Fig. 30. Elution profile of human factor $\bar{D}$. EDTA-treated human serum was passed over a Sephadex G-75 column equilibrated in 0.15 M NaCL, 0.002M EDTA, pH 7.3. Fractions were assayed for optical density at 280nM.

alternative pathway activation, whereas the lipid A moiety is responsible for activation of the classical pathway. These results require confirmation utilizing purified lipopolysaccharide and lipid A prepared from E. coli 075. However, they suggest that microorganisms such as E. coli 075 with complete polysaccharide regions in their cell walls would activate the alternative pathway under normal conditions, since this region of the gram-negative cell wall is outermost. The lipid A moiety is the innermost portion of the lipopolysaccharide and is located directly next to the muco-peptide layer of the cell wall. The classical pathway would therefore not be activated unless the cell wall was altered in some manner as to expose the lipid A or unless the alternative pathway was unable to be utilized. (c) Heat-stable immune IgG antibodies to E. coli 075 which would normally trigger classical pathway activation were not demonstrated in either normal or burn sera. In fact, the data suggest a minimal role, if any, for immunoglobulin in opsonization of E. coli 075. These later observations confirm the results of Jasin who also demonstrated that opsonization of E. coli 075 proceeded normally in immunoglobulin depleted human serum (31).

The tentative conclusion that alternative pathway proteins required for opsonization of E. coli 075 were abnormal in the burn sera requires confirmation by experimental studies. This possibility is extremely interesting, however, in light of our previous observation that C3 conversion by inulin in the burn sera was normal when E. coli opsonization and classical pathway activity were reduced. Inulin is a recognized activator of the alternative pathway, and all of the studies which are currently available on the protein interactions of the alternative pathway have utilized zymosan or inulin as the activating substance. Our data would indicate that when bacteria, such as E. coli 075, are used as the activating substances, the protein interactions involved in alternative pathway activation may differ from the protein interactions involved when inulin is used as the activating substance. The sequence of recognized protein interactions

may be different or proteins in addition to factors B, $\overline{D}$, and initiating factor may be required to generate an alternative pathway C3 convertase by the bacteria. These possibilities emphasize the importance of future studies to determine the identity and sequence of proteins required for opsonization of gram-negative aerobes such as E. coli 075.

Our study also showed that gram-negative aerobic bacilli, in addition to E. coli 075, were capable of activating the alternative pathway. Efficient alternative pathway activation was achieved utilizing intact cells of P. aeruginosa, P. mirabilis, and S. minnesota S form. S. aureus, a microorganism which frequently causes serious infections in burned patients, was not found to be as efficient as the gram-negative bacilli in activating the alternative pathway, although this microorganism appeared highly active in initiating classical pathway activation. The immunological specificities of S. aureus reside in the polysaccharide A antigen which is teichoic acid composed of N-acetyl glucosamine residues attached to a polyribitol phosphate backbone (67, 68). Most strains of S. aureus also possess the surface component, protein A, which reacts with the Fc fragments of the IgG molecules in most mammalian sera (69-72). Since isolated protein A is known to fix complement, the interaction of IgG with protein A of the cell wall of S. aureus may promote binding of Clq and thus preferential activation of the classical pathway (73). Our future studies in this area will be directed toward determining if strains of gram-negative bacilli isolated from burned patients activate the alternative pathway as effectively as our test strains.

The data obtained in this investigation have also aided in the interpretation of some of our unexplained previous observations regarding changes in humoral factors in burned patients. In this regard, inhibition of formation of an alternative pathway C3 convertase which we have demonstrated in burned

patients may be of critical importance to the patient if classical pathway activity is decreased. Our results suggest that each complement pathway should be able to compensate for the other, if either is not functioning properly. If the alternative pathway is unable to be activated due to an inhibitory factor and classical pathway components are consumed during septicemia, then opsonization of the patient's sera for his infecting microorganism should be reduced if the organism requires complement activation for opsonization. Our results presented in section A1 of this report support this thesis.

Another interesting possibility which is supported by this investigation is that there may be more than one way to activate the classical pathway. It is well known that immune IgG antibacterial antibodies can bind the C1q portion of C1 and thereby initiate the rest of the classical sequence. Our results support the concept that classical pathway activation can also occur in the absence of antibody. Loos et al (63) and quite recently Morrison and Kline (74) have provided evidence to suggest that the lipid A region of the lipopolysaccharide molecule interacts directly with C1 to initiate classical pathway activation by a mechanism which does not require antibody.

2. Studies to determine the requirement for immunoglobulin and complement in opsonization of B. fragilis

a. Results

In our previous studies, methodology was developed for measuring the in vitro interaction of human leukocytes and serum factors in phagocytosis and intracellular killing of gram-negative non-sporulating anaerobes (75). Strains of B. fragilis subspecies fragilis and thetaiotaomicron were shown to be killed by human leukocytes in the presence of pooled normal human serum (PNHS), but not by either leukocytes or serum alone. The present investigation was undertaken to define the role of immunoglobulin and complement in phagocytosis and intracellular killing of the B. fragilis strains by leukocytes. All of the experiments to be described were carried out under anaerobic conditions as previously detailed (75).

In our preliminary experiments, a standard anticomplementary procedure (heating at 56°C for 30 minutes) was used to inactivate complement in PNHS. In addition, hypogammaglobulinemic sera from three different donors were used as sources of immunoglobulin depleted sera. Neither the hypogammaglobulinemic sera nor the heated PNHS supported phagocytosis and intracellular killing of the B. fragilis strains (Fig.31). In contrast, untreated PNHS promoted over a one log reduction in bacterial counts of both subspecies of B. fragilis. These results indicated that both complement and immunoglobulin in normal human serum were required for opsonization of B. fragilis subspecies fragilis and thetaiotaomicron.

To determine the participation of late-acting complement components, use was made of C8 deficient human serum and C6 deficient rabbit serum. The opsonic activity of the C8 deficient human serum was as efficient as PNHS (Fig.32). The opsonic activity of the C6 deficient rabbit serum could not be evaluated,

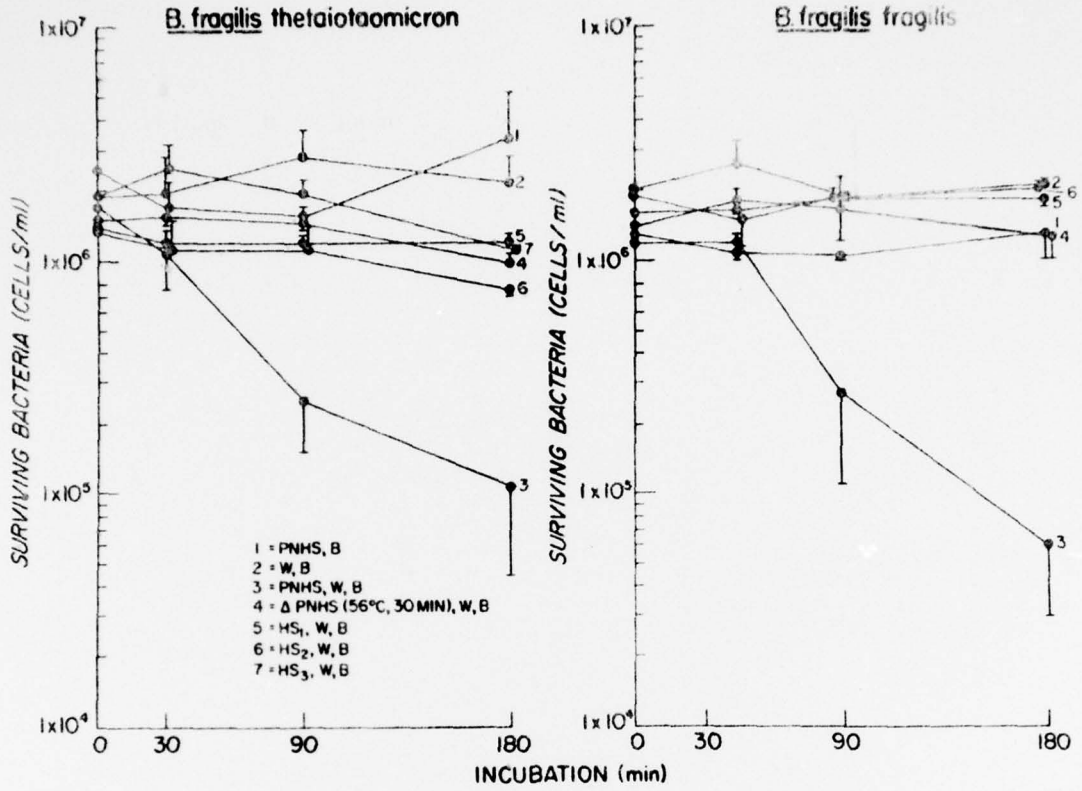

Fig. 31. Opsonic activity of human sera depleted of complement or immunoglobulin for B. fragilis subspecies fragilis (#1365) and thetaiotaomicron (#1343). In the left figure, experiments were performed using strain #1343 and in right figure, experiments were performed using strain #1365. The following reaction mixtures were tested: (1) pooled normal human serum (PNHS) and bacteria; (2) leukocytes and bacteria; (3) PNHS, leukocytes, and bacteria; (4) heated PNHS (56°C, 30 minutes), leukocytes, and bacteria; (5) hypogammaglobulinemic serum (HS_1), leukocytes, and bacteria; (6) HS_2, leukocytes, and bacteria; (7) HS_3, leukocytes, and bacteria. The hypogammaglobulinemic sera contained 240 to 330 mg% of IgG, 16 to 29 mg% of IgM, and less than 24 mg% of IgA. The points represent mean values of 2 to 5 experiments, and the vertical bars represent the standard error of the mean.

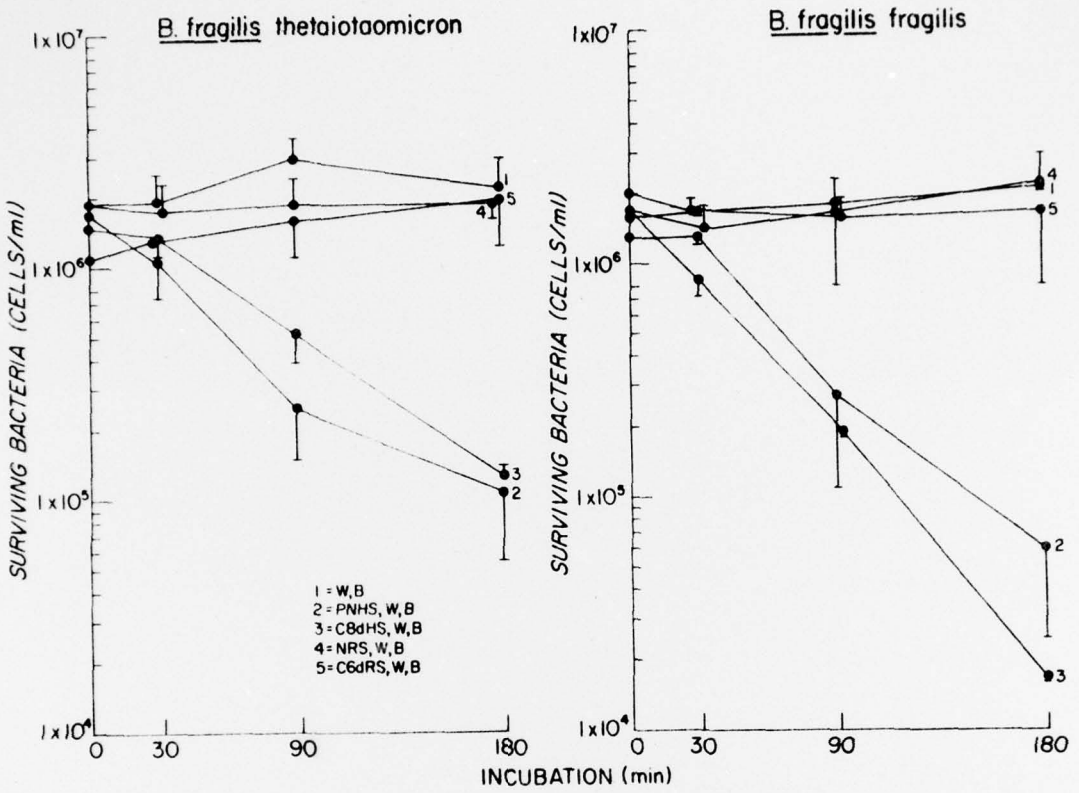

Fig. 32. Opsonic activity of C6 and C8 deficient sera for B. fragilis subspecies fragilis (#1365) and thetaiotaomicron (#1343). In the left figure, experiments were performed using strain #1343 and in the right figure experiments were performed using strain #1365. The following reaction mixtures were tested: (1) leukocytes and bacteria; (2) PNHS, leukocytes, and bacteria; (3) C8 deficient human serum, leukocytes, and bacteria; (4) normal rabbit serum, leukocytes, and bacteria; (5) C6 deficient rabbit serum, leukocytes, and bacteria. The points represent mean values of 2 to 5 experiments, and vertical bars represent the standard error of the mean.

since normal rabbit serum was also found to be unable to promote phagocytosis and killing of the test strains. These results indicated that C8 and C9 were not required for opsonization of the B. fragilis strains.

To determine the participation of the alternative complement pathway in opsonization of the bacteria, the ability of the strains to be phagocytosed and killed by leukocytes in the presence of sera depleted of components of this pathway was next investigated. Human sera depleted of terminal complement components by inulin or cobra venom factor (CoVF), recognized activating substances of the alternative pathway, were unable to support phagocytosis of the test strains (Fig.33). The opsonic activity of C4 deficient guinea pig serum was unable to be evaluated, since the maximum reduction in bacterial counts in the presence of normal or C4 deficient sera was only 0.5 log. PNHS depleted of C3 by treatment with 0.15M hydrazine at 37°C for 60 minutes (R3) was also unable to effecti psonize the test strains (Fig.34). In addition, the opsonic activity of as markedly reduced by heat treatment at 50°C for 30 minutes which removes factor B (RB) and by depleting the serum of factor $\bar{D}$ by molecular sieve chromatography on Sephadex G-75 (RD). The only differences between the opsonic requirements of the two subspecies were observed when the opsonic activity of properdin depleted serum (RP) was tested. PNHS was depleted of properdin by treatment with zymosan at 17°C in the presence of magnesium ions. The opsonic activity of RP for the subspecies fragilis was equivalent to the opsonic activity of PNHS. The kinetics of opsonization of the subspecies thetaiotaomicron by RP was markedly decreased, and a significant reduction in bacterial counts was only observed after 3 hours of incubation. The results provide indirect evidence to indicate an absolute requirement for C3 and factors B and $\bar{D}$ for opsonization of both B. fragilis strains and a partial requirement for properdin for opsonization of the subspecies thetaiotaomicron,

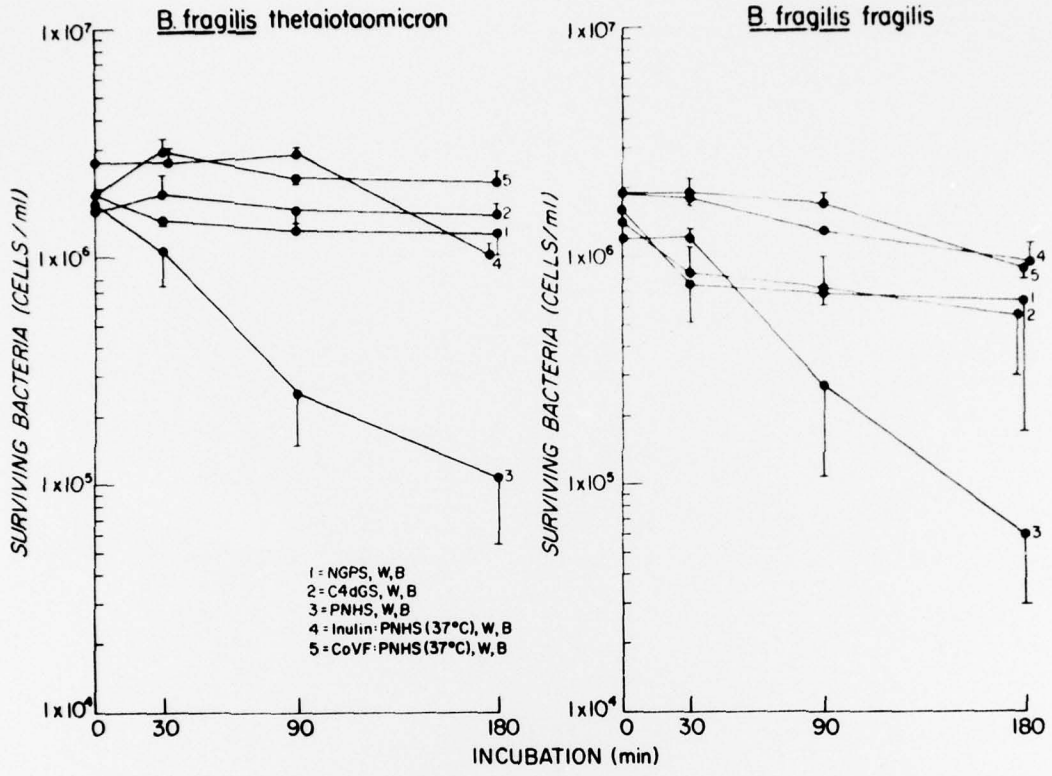

Fig. 33. Participation of the alternative complement pathway in opsonization of B. fragilis subspecies (#1365) and thetaiotaomicron (#1343). In the left figure, experiments were performed using strain #1343 and in right figure, experiments were performed using strain #1365. The following reaction mixtures were tested: (1) normal guinea pig serum, leukocytes, and bacteria; (2) C4 deficient guinea pig serum, leukocytes, and bacteria; (3) PNHS, leukocytes, and bacteria; (4) PNHS treated with 10 mg/ml of inulin (37°C, 60 minutes), leukocytes, and bacteria; (5) PNHS treated with 10 units per ml of CoVF (37°C, 60 minutes), leukocytes, and bacteria. The points represent mean values of 2 to 5 experiments, and the vertical bars represent the standard error of the mean.

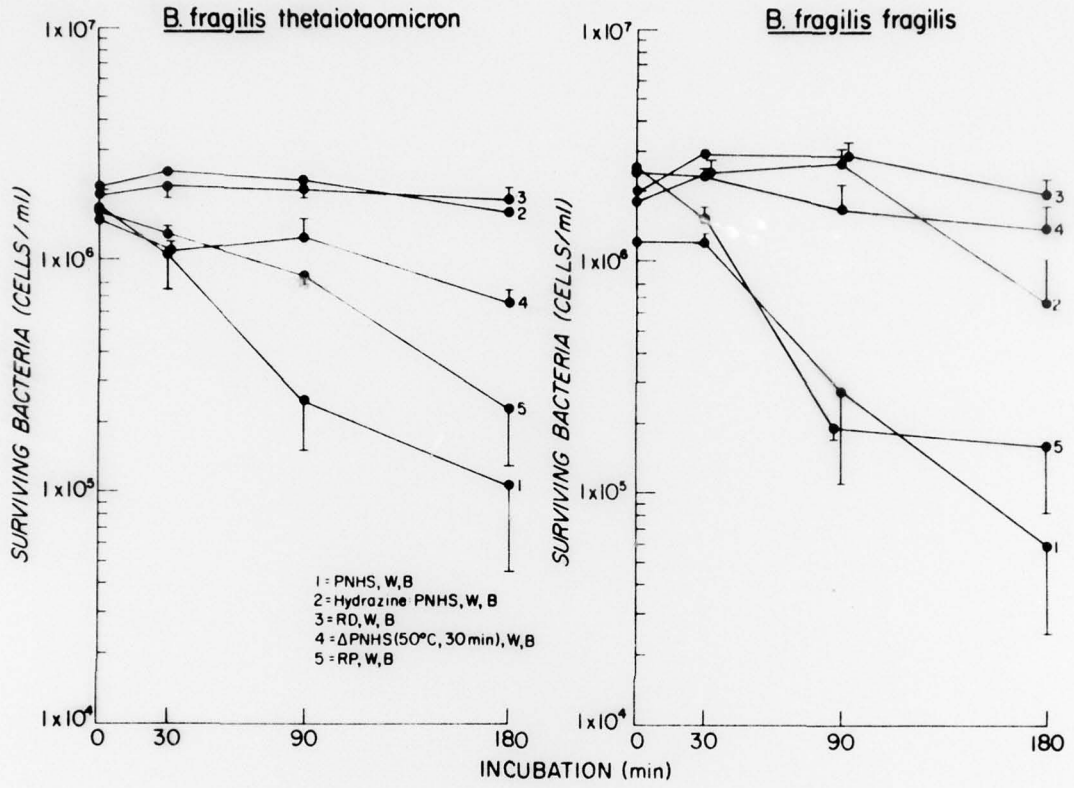

Fig. 34. Opsonic activity of PNHS depleted of alternative pathway components for B. fragilis subspecies fragilis (#1365) and thetaiotaomicron (#1343). In the left figure, experiments were performed using strain #1343 and in the right figure, experiments were performed using strain #1365. The following reaction mixtures were tested: (1) PNHS, leukocytes, and bacteria; (2) R3, leukocytes, and bacteria; (3) RD, leukocytes, and bacteria; (4) RB, leukocytes, and bacteria; (5) RP, leukocytes and bacteria. The points represent mean values of 2 to 5 experiments, and the vertical bars represent the standard error of the mean.

but not for opsonization of the subspecies fragilis.

Since heat treatment at 50°C for 30 minutes is known to inactivate C2 as well as factor B, heated serum was supplemented with purified human C2 or factor B and opsonic activity was determined. Addition of two different preparations of purified human C2 to heated PNHS at a concentration which has been shown to restore hemolytic activity to normal did not substantially increase its opsonic activity for the bacterial strains (Fig.35). Opsonic activity of the heated serum was fully restored to normal by physiological concentrations of two different preparations of purified human factor B (FB_1 and FB_2). These results indicate a requirement for factor B for opsonization of the B. fragilis strains.

b. Discussion

In the present investigation, evidence was provided to indicate a requirement for immunoglobulin and components of the alternative complement pathway in normal human serum for opsonization of B. fragilis subspecies fragilis and thetaiotaomicron. This represents a totally new observation, since there is currently no literature available regarding the definition of serum proteins required for opsonization of the gram-negative non-sporulating anaerobes.

The experimental data in support of this preliminary conclusion are as follows: (a) Human sera depleted of immunoglobulin, C3, factors B or D or terminal complement components C3 to C9 did not support phagocytosis and intracellular killing of the microorganisms by normal human leukocytes. (b) The opsonic activity of human serum heated at 50°C for 30 minutes for the B. fragilis strains was restored to normal by highly purified factor B, but not by human C2, the component of the classical pathway which is labile at 50°C. (c) Properdin depleted human serum supported normal phagocytosis of the B. fragilis fragilis and only partially supported phagocytosis of the subspecies thetaiotaomicron, suggesting participation but not an absolute requirement for properdin

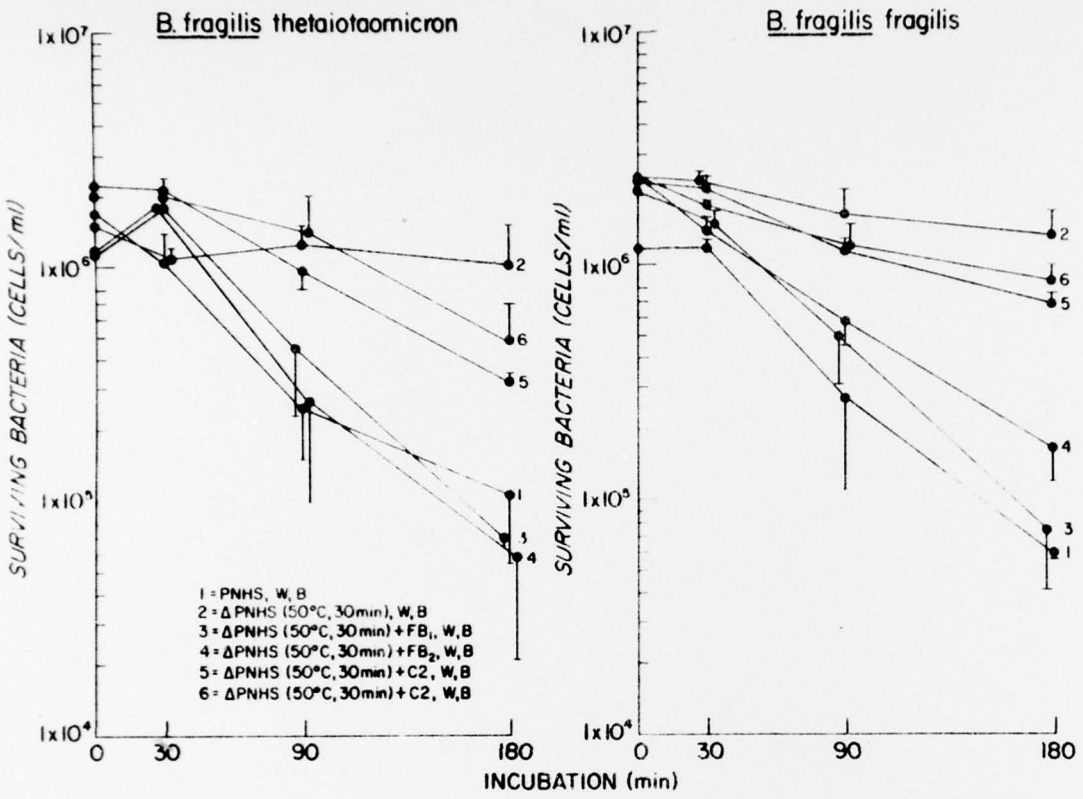

Fig. 35. Restoration of the opsonic activity of heated PNHS by purified factor B. In the left figure, experiments were performed using strain #1343 and in the right figure, experiments were performed using strain #1365. The following reaction mixtures were tested. (1) PNHS, leukocytes, and bacteria; (2) △ PNHS (50°C, 30 min.), leukocytes, and bacteria; (3) ◿ PNHS supplemented with 30 µg/ml of FB_1, leukocytes and bacteria; (4) △ PNHS supplemented with 42 ug/ml of FB_2, leukocytes and bacteria; (5) Δ PNHS supplemented with 100 units/ml of $C2_1$, leukocytes, and bacteria; (6) ◿ PNHS supplemented with 100 units/ml of $C2_2$, leukocytes, and bacteria. The points represent mean values of 2 to 5 experiments, and the vertical bars represent the standard error of the mean.

for opsonization of the thetaiotaomicron subspecies. This observation is not surprising, since C3 conversion and thus presumably opsonization can occur in the absence of properdin. The function of properdin is to stabilize the C3 convertase, $\overline{C3b,B}$ formed by the feedback mechanism involving factors B, $\overline{D}$, and C3b (Refer to Fig.1).

In our investigation, immunoglobulin in addition to components of the alternative complement pathway was found to be required for opsonization of the B. fragilis strains. Available information regarding the role of immunoglobulin in opsonization of microorganisms is controversial. Antibody of the IgG class, in addition to components of the alternative complement pathway, has been shown to participate in opsonization of P. aeruginosa (26,27) and Streptococcus pneumoniae (28). In contrast, opsonization of strains of E. coli (31), S. epidermidis, S. aureus, S. marcescens, S. viridans, and S. faecalis (76) has been shown to proceed normally in hypogammaglobulinemic sera.

The question of the role of immunoglobulin in activation of the alternative pathway is also unanswered. Available evidence indicates that activation by inulin or zymosan occurs in the absence of immunoglobulin (18). In addition, lysis of Trypanosoma cyclops (77) and rabbit erythrocytes (78) by normal human serum has been shown to require activation of the alternative pathway without an apparent requirement for conventional antibodies. On the other hand, antibody of the IgG class has been shown to participate in alternative pathway mediated lysis of measles virus infected cells (79) as well as in opsonization of bacteria as described above.

Since complement activation is required for opsonization of bacteria, one of the most important questions in this research area remaining to be answered is whether immunoglobulin is required directly for activation of the alternative pathway or for binding of the bacterium to receptors on the leukocyte cell membrane surface. The experimental design of our renewal application will

include a comprehensive study focused on answering this as yet unresolved issue.

The results of our investigation indicate that the human serum proteins required for opsonization of B. fragilis subspecies fragilis and thetaiotaomicron were similar if not identical. This finding raises the question as to the importance of the polysaccharide capsule to the virulence of B. fragilis. Kasper has suggested that the polysaccharide capsule found almost exclusively on the fragilis subspecies may act as an antiphagocytic agent (80). Our results would tend to support the thesis that the antiphagocytic cell surface component is not unique to the subspecies fragilis and probably may not be the primary virulence factor of this microorganism. Alternatively, our strain of B. fragilis subspecies thetaiotaomicron may be encapsulated although this possibility is considered unlikely, since Kasper has only found one of the several strains of these subspecies to be encapsulated. Our future studies will attempt to examine the opsonic requirements for additional strains of B. fragilis subspecies fragilis and thetaiotaomicron. Of additional importance will be to examine the interaction of human serum and leukocytes with strains of B. fragilis vulgatus and distasonis, since none of these strains has been shown to contain capsular material.

V. CONCLUSIONS

A. Consumption of components of the classical complement pathway was associated with and was probably caused by septicemia in thermally injured patients.

B. Decreased classical pathway activity was demonstrated during the first week postburn in all burned patients who subsequently developed septicemia.

C. C3 conversion via the alternative pathway was also reduced in some of the burned patients during septic episodes and appeared to be due to inhibition rather than to consumption of proteins required for C3 conversion.

D. In one patient, consumption of components of the classical complement pathway occurring during septicemia decreased the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of E. coli. In the other patients, consumption of classical pathway components did not reduce the opsonic capacity of the patients' sera for their infecting strains all of which, with one exception, were staphylococci.

E. All of the microorganisms isolated from the burned patients were not susceptible to complement mediated lysis and were phagocytosed and killed intracellularly by human leukocytes only in the presence of human serum. The only exception were the C. albicans strains isolated from the burned patients which were not phagocytosed and killed intracellularly by leukocytes even in the presence of high concentrations of normal human serum.

F. Preliminary evidence was provided to suggest that reduction in C3 conversion via the alternative pathway occurring in burned patients during the tenth to 60th postburn days was caused by elevation of a normal regulatory protein of the complement system.

G. Abnormalities of both the alternative and classical complement pathways and of immunoglobulin M were found to occur immediately following severe blunt, or penetrating abdominal trauma.

H. No reduction in immunochemical levels or functional activity of components of the classical or alternative complement pathways or of immunoglobulins were demonstrated in septic patients without trauma, suggesting that complement consumption in the septic burned patients was a result of synergism between the infection and the trauma.

I. Intact cells of E. coli 075, wild-type S. minnesota, and clinical isolates of P. aeruginosa and P. mirabilis, were found to be capable of efficiently activating the alternative complement pathway. S. aureus was less efficient in activating the alternative pathway, although this microorganism was highly active in initiating classical pathway activation.

J. Utilizing washed cells of S. minnesota cell wall mutants, the polysaccharide portion of the lipopolysaccharide was shown to be responsible for alternative pathway activation, whereas the lipid A moiety was responsible for activation of the classical pathway.

K. Factor $\overline{D}$ was obtained from normal human serum in partially purified form.

L. Heat-stable immune IgG antibodies to E. coli 075 were not demonstrated in either normal or burn sera, and opsonization of E. coli 075 was shown to proceed normally in immunoglobulin depleted sera.

M. Immunoglobulin and components of the alternative complement pathway in normal human serum were shown to be required for phagocytosis and intracellular killing of B. fragilis subspecies fragilis and thetaiotaomicron by human peripheral leukocytes.

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